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TITLE: A Medical Research and Evaluation Facility and Studies Supporting the Medical Chemical Defense Program

SUBTITLE: Time and Dose Response Characterization of the Prevention of HD-Induced NAD+ Depletion and HD-Induced Cytotoxicity by Niacinamide and Niacin

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13. ABSTRACT *(Maximum 200 words)*

Studies were conducted under this task to Assess the time and concentration dependent nature of niacinamide (NM) protection against HD-induced NAD^+ depletion and cytotoxicity. The HD concentrations used assess the time dependent nature of cytotoxicity and NAD^+ depletion, and the impact of NM and niacin (NI) treatment were 13, 62, 101, and 171 μM HD. Three concentrations of NM and NI were (0.01, 0.1 and 1 mM) selected by USAMRICD for evaluation at 2, 4, 8, 12, 16, 20, 24, 48, and 72 hours after exposure to HD. Cytotoxicity and total culture NAD^+ content were assessed.

NAD^+ concentrations following the addition of 1 mM NM frequently were significantly greater than those observed for the HD-exposed controls especially at 171μM HD. Multiple addition of NM had little protective effect relative to that by provided by pretreatment alone. At the 171μM HD concentration, the single addition of NM provided marginal but statistically significant ($p \leq 0.05$) protection. Comparisons of the different NM addition modes yielded mixed results, but usually the number of viable cells was greater with multiple additions of NM. NI did not provide protection against HD-induced NAD^+ depletion or cytotoxicity.

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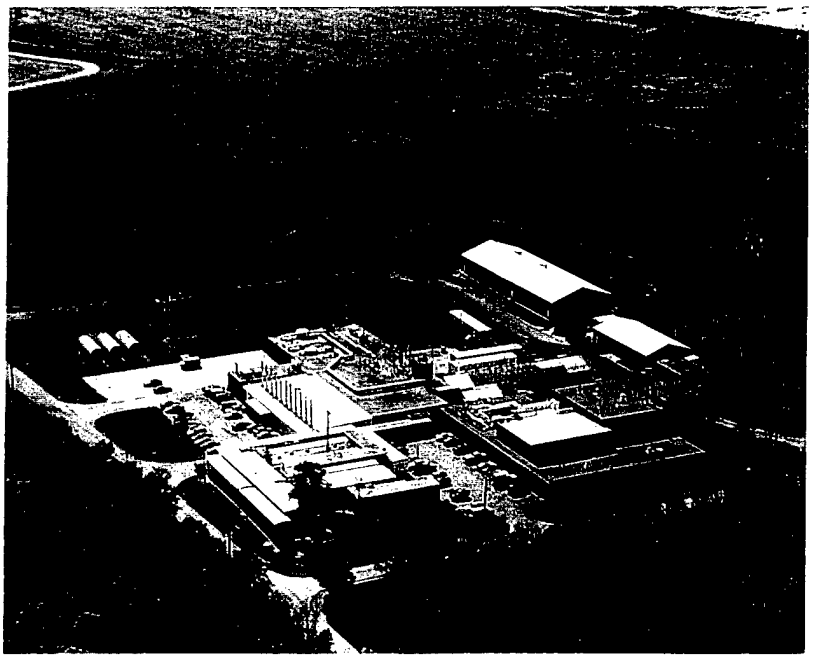

Task 91-22 TIME-AND DOSE-RESPONSE CHARACTERIZATION OF THE PREVENTION OF HD-INDUCED NAD+ DEPLETION AND HD-INDUCED CYTOTOXICITY BY NIACINAMIDE AND NIACIN

To

U.S. Army Medical Research and Development Command

January, 1997

Battelle
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EXECUTIVE SUMMARY

A research objective of prime importance to the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) is the characterization of the pathophysiology produced by sulfur mustard (HD) in order to design effective therapeutic interventions. One hypothesis of HD pathophysiology is centered on the poly(ADP-ribose) polymerase (PADPRP) enzyme, which is activated by HD-induced deoxyribonucleic acid (DNA) damage. Once activated, PADPRP rapidly depletes cellular nicotinamide adenine dinucleotide (NAD^+), leading to metabolic alterations, protease release, cell death, and ultimately vesication (Papirmeister et al., 1985).

Prior to initiating this project in late 1991, studies had been conducted which indicated that niacinamide (NM), an inhibitor of PADPRP activity, provided protection against HD-induced cytotoxicity in lymphocytes and human epidermal keratinocytes (HEKs) in cultures (Meier et al., 1987; Smith et al., 1990). HD-induced NAD^+ depletion in human skin transplanted onto nude mice and in cultured human skin could also be ameliorated by NM (Gross et al., 1985; Mol et al., 1991). Although NM could provide protection against HD-induced NAD^+ depletion in cultures of human skin, NM did not alter the incidence of HD-induced microvesicle formation (Mol et al., 1991). Studies performed with hairless guinea pigs, however, indicated that NM reduced the incidence of microvesication at 24 hr, but not at 72 hr following HD exposure unless NM was given prior to HD (pretreatment) as well as multiple times after HD treatment (Yourick et al., 1991). Initial studies with HEK cultures indicated similar findings in that NM provided protection against HD-induced cytotoxicity measured at 24 hr following exposure, but protection was lost when measured at extended time periods of incubation (Smith et al., 1991). Since PADPRP inhibitors prevent HD-induced NAD^+ depletion, the protective effect against cytotoxicity was presumed to be due to maintenance of cellular NAD^+ levels (Smith et al., 1991; Meier et al., 1987).

Studies were conducted under this task to assess the time and concentration dependent nature of NM protection against HD-induced NAD^+ depletion and cytotoxicity. As HD-induced NAD^+ depletion and cytotoxicity occur within 24 hr of exposure, the majority of

DTIC QUALITY INSPECTED 8

measurements were planned for the first 24 hr, with measurements also at 48 and 72 hr. Niacin (NI), which like NM serves as a precursor for NAD^+ synthesis, but does not inhibit PADPRP activity, was evaluated as a control. Initial studies assessed the relationship between HD exposure and NAD^+ depletion and cytotoxicity at 24 hr following exposure to determine HD concentrations that could be used in subsequent studies to assess the time-dependent onset of effects in the presence and absence of NM and NI. HD caused no observable effect, 25 percent, 50 percent, and 75 percent reduction in total culture NAD^+ content at approximately 24 hr following exposure at 13, 62, 101, and 171 μM, respectively. For cytotoxicity, as assessed by propidium iodide (PI) exclusion from cells with intact membranes (viable cells), 25, 50, and 75 percent cell deaths were estimated to occur at 69, 114, and 189 μM HD, respectively.

The HD concentrations used for studies to assess the time-dependent nature of cytotoxicity and NAD^+ depletion, and the impact of NM and NI treatment, were those derived from the 24 hr NAD^+ depletion data (i.e., 13, 62, 101, and 171 μM HD). In these studies, NM and NI were either added as a pretreatment only, or added as a pretreatment and then added again at 2, 24, and 48 hr as a treatment. Three concentrations of NM and NI (0.01, 0.1, and 1 mM) were selected by USAMRICD for evaluation. At 2, 4, 8, 12, 16, 20, 24, 48, and 72 hr following exposure to the various HD concentrations, cytotoxicity and total culture NAD^+ content were assessed.

Average NAD^+ concentrations following the addition of 1 mM NM frequently were significantly greater than those observed for the HD-exposed controls, especially at 171 μM HD. Multiple addition of NM had little protective effect relative to that provided by the pretreatment alone. Comparisons of NM or NI pretreated cells with HD-exposed control cells usually showed insignificant protection from cell death at the 101 μM HD level, where toxicity was observed by 24 hr of exposure. At the 171 μM HD level, the single addition of NM provided marginal but statistically significant ($p \leq 0.05$) protection. Comparisons of the different NM addition modes yielded mixed results, but usually the number of viable cells was greater when there were multiple additions of NM. Regardless of addition mode, NI did not provide protection against HD-induced NAD^+ depletion or cytotoxicity. Data from time

course studies of NAD^+ content in control cultures indicated increasing NAD^+ levels over time. This increase could reflect a change in NAD^+ content of cells as the cultures reach confluence and/or be indicative of increased cell number (Jacobsen and Jacobsen, 1980).

After discussing the data with the USAMRICD Technical Point of Contact, studies were conducted to assess protein content in cultures as a means of standardizing biochemical data. Cells were exposed to the four HD concentrations previously used. At 0, 4, 24, 48, and 72 hr, cellular protein content, cell number, and cell viability were assessed. At 24 hr, control culture cell number had increased 1.2-fold the time zero value. Both cell number and protein content in control cultures increased 1.8-fold the time zero value by 72 hr. HD concentrations as low as 13 μM inhibited the increase in cell number, yet protein concentration continued to increase over the 72 hr period, possibly indicating asynchronous cell growth. At higher HD concentrations, cell numbers decreased below baseline levels, indicating that proliferation was inhibited and that cell lysis may have occurred. At these higher HD concentrations, the fraction of intact, nonviable cells measured by cytofluorimetry did not increase, and the amount of protein per cell was elevated. At the highest HD concentration, 171 μM, cell loss and an increased number of nonviable cells were observed. Unlike the lower HD concentration groups, the amount of protein on a per cell basis was not elevated. Although these data indicated that protein may not be a suitable indicator of cell number for standardization purposes, data in the literature indicates that the amount of protein per unit cell volume does not change in cells exposed to DNA synthesis inhibitors (Cohen and Studzinski, 1967).

NM provided marginal to no protection against HD-induced toxicity in HEK as measured by the PI exclusion method. The cell number data demonstrate the anti-proliferative effect of HD and the need to standardize biochemical data for cultures proliferating following HD exposure. Although these studies indicate that protein content may not accurately assess cell number, it may be an accurate assessment of cell volume.

As directed by the USAMRICD Contracting Officer's Representative (COR) in February 1996, task closeout was initiated.

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TIME- AND DOSE-RESPONSE CHARACTERIZATION OF THE PREVENTION OF HD-INDUCED NAD^+ DEPLETION AND HD-INDUCED CYTOTOXICITY BY NIACINAMIDE AND NIACIN

1.0 INTRODUCTION

Dermal exposure to HD can produce incapacitating skin injuries with the hallmark of damage being the formation of large, fluid-filled blisters that tend to heal rather slowly (Eisenmenger et al., 1991). Consistent with the radiomimetic nature of the systemic toxicity induced by HD on human bone marrow, lymphoid tissue, and gastrointestinal epithelium, the basal keratinocytes of human skin have been shown to be sensitive to HD-induced toxicity and this toxicity is thought to be penultimate to vesication (Calabresi and Chabner, 1990; Papirmeister et al., 1984). Despite years of research and several hypotheses, the precise mechanism of HD-induced cell toxicity and vesication is unknown (Papirmeister, 1994).

A hypothesis forwarded in the mid 1980's to explain HD-induced pathophysiology of basal keratinocytes is centered on poly(ADP-ribose) polymerase (PADPRP) activation following HD-induced DNA damage (Papirmeister et al., 1985). DNA damage is associated with the activation of PADPRP in mammalian cells (Berger et al., 1979; Sims et al., 1982; Carson et al., 1986). PADPRP activity uses nicotinamide adenine dinucleotide (NAD^+) irreversibly as it transfers the ADP-ribose moiety of NAD^+ to nuclear proteins or to other ADP-ribose functional groups, forming a poly(ADP-ribose) chain. Elevated activity of PADPRP is associated with excision-repair of damaged DNA. If sufficient activation occurs, cellular NAD^+ rapidly becomes depleted with subsequent alterations of cellular metabolism and energy production, resulting in cell death (Carson et al., 1986). NAD^+ depletion has been demonstrated to occur within a few hours of HD exposure *in vitro* and *in vivo* (Meier et al, 1987; Gross et al., 1985; Smith et al., 1992; Yourick et al., 1991). HD-induced NAD^+ depletion, with ensuing effects on cellular metabolism and basal keratinocyte cell death, is postulated to cause the release of intracellular proteases leading to vesication. Studies to evaluate this hypothesis have utilized different model systems as well as different inhibitors of PADPRP activity, and have provided inconclusive evidence in support of this hypothesis (Papirmeister, 1994).

Support for this hypothesis came through the use of niacinamide (NM), which is a precursor in NAD^+ synthesis and also an inhibitor of PADPRP activity. Addition of NM to cultures of human leukocytes has been shown to ameliorate HD-induced NAD^+ depletion and cytotoxicity (Meier et al., 1987). Similarly, niacin (NI) serves as a precursor in NAD^+ synthesis and also was shown to prevent cytotoxicity, as did 3-methoxybenzamide, an inhibitor of PADPRP activity (Meier et al., 1987). Studies performed with hairless guinea pigs also indicated that NM treatment might reduce some of the skin damage induced by HD (Yourick et al., 1991).

NM treatment of HD-exposed human skin cultures prevented NAD^+ depletion, but did not prevent basal cell toxicity or microblister formation, indicating an alternative or additional mechanism for vesication (Mol et al., 1991). Data from *in vitro* studies using proliferating human epidermel keratinocytes (HEK) showed mixed results with respect to protection with NM from HD-induced toxicity (Smith et al., 1991, Mol et al., 1989). While NM provided protection against HD-induced cell death as assessed by PI uptake, NM did not protect against HD-induced reduction in glucose uptake by HEK. In some proliferating cells, PADPRP inhibitors have been shown to actually enhance the toxicity induced by DNA damaging agents (Jacobson et al., 1985; Lunec et al., 1984; Schwartz et al., 1985; Ben-Hur et al., 1985).

Precursors in the synthesis of NAD^+ (e.g., NI and NM) and inhibitors of PADPRP activity (e.g., NM) are considered potential therapeutic compounds for HD exposure. *In vivo* studies with hairless guinea pigs and *in vitro* studies with HEKs have indicated that NM can reduce the incidence of microblister formation and cytotoxicity at 24 hr following exposure, but this protective effect is lost at 72 hr following exposure (Yourick et al., 1991; Smith et al., 1991). To further investigate the relationship between NAD^+ levels, cytotoxicity, and PADPRP activation, Battelle was tasked to characterize the time- and concentration-response relationships between HD, NM and NI in a widely used cell culture system to further evaluate the hypothesis.

2.0 MATERIALS AND METHODS

HD was supplied by The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), along with data regarding its purity, identification (lot number), and stability. The lot number of HD used in these studies was U-6216-CTF-N-1, HI-57 (lot 6). HD was analyzed at Battelle's Medical Research and Evaluation Facility (MREF) each time the vial was opened, and monthly during storage. HEKs (Strain No. 2041-1), growth medium, trypsin-ethylenediamine tetracetic acid (trypsin-EDTA) solution, and trypsin neutralizing solution were obtained from Clonetics Corporation (San Diego, CA). Latex beads for standardizing the flow cytometer forward scatter detector were purchased from Flow Cytometry Standards Corporation (Research Triangle Park, NC). Tritiated-NAD was purchased from ICN (Costa Mesa, CA) and DuPont/NEN (Boston, MA). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

2.1 Cell Model

The cell model used in these studies was the HEK, and cultures were obtained as cryopreserved primary cells from Clonetics Corporation. These cells were cultured following MREF Method No. 16/In Vitro (See Appendix B). Cells were seeded at 2,500/cm^2 in 25-cm^2 tissue culture flasks. When these secondary cultures were approximately 80 percent confluent, the cells were detached using trypsin-EDTA, and tertiary cultures started at 2,500 cells/cm^2 in either 25-cm^2 tissue culture flasks for evaluating cellular growth kinetics, 96-well microplates for NAD^+ assays, 24-well plates for cytotoxicity assays, or 6-well microplates for simultaneous measurement of cell number, protein content, and cytotoxicity. Culture confluence was assessed by microscopic observation (40X magnification).

2.2 HD Exposure

Cell cultures were exposed to levels of HD following Battelle Standard Operating Procedure (SOP) MREF V-011 (See Appendix B). The total volume of cultures contained in 96-well, 24-well, and 6-well microplates was 0.1, 1, and 3 mL, respectively. Following

exposure to HD, microplates were stored in a biological safety cabinet for 1 hr at room temperature to allow HD hydrolysis while providing a mechanism for HD vapors to be vented. This one hour was considered part of the exposure time. The microplates were then transferred to a controlled environment incubator (5 percent carbon dioxide and 37 degrees C) for the designated period.

2.3 NM and NI Additions

When utilized, NM and NI were added 15 to 30 min prior to the addition of HD. When no further compound was added to cultures, this was termed single addition. For the multiple addition regimen, compound was added to cultures again at 4, 24, and 48 hr. For this process, 10 μL of NM (0.1, 1, or 10 mM) or 10 μL of NI (1, 10, or 100 mM) was added to cultures contained in 96-well plates destined for NAD^+ analyses. For the cytotoxicity assay, cultures were centrifuged at 400 x g for 5 min, 100 μL of supernatant was removed, and 100 μL of NM or NI was added to obtain final concentrations of 0.01, 0.1, or 1mM for NM or 0.1, 1, or 10 mM for NI.

2.4 HD Concentration Responses

Tertiary HEK cultures contained in 96-well microplates (NAD^+ depletion) or 24-well microplates (cytotoxicity) were exposed to various amounts of HD for assessing the effects on NAD^+ and viability at 24 hr following HD addition.

NAD^+ values were converted to percent NAD^+ depletion and cytotoxicity data was standardized relative to a vehicle control. Data from all experimental runs were analyzed collectively using a three-parameter probit model:

$$p = G * \Phi(\alpha + \beta * \log_{10}[HD])$$

where:

α = Y - intercept of the HD concentration-response relationship
β = slope of the concentration-response relationship
G = maximal response
p = percent response exhibited at $\log_{10}[HD]$
Φ = cumulative distribution function for the normal distribution.

Estimated parameters from the HD concentration-response data set were used to calculate the HD concentrations required to produce 25 percent (IC_{25}), 50 percent (IC_{50}), and 75 percent (IC_{75}) responses. These parameters also were used to estimate the highest HD concentration which had a lower 95 percent confidence limit equal to or less than zero. This estimated HD concentration was considered the no observable effect level (NOEL) for HD. Confidence intervals for percentiles of the HD concentration-response distribution were computed using Fieller's method.

2.5 NM and NI Evaluations

Tertiary HEK cultures contained in 96-well microplates (NAD^+ depletion) or 24-well microplates (cytotoxicity) were used for assessing the effect of NM and NI pretreatment/treatment on HD-induced NAD^+ depletion and cell death. Cultures received either a single addition, or multiple additions of NM and NI. Cultures were exposed to HD concentrations of 0, 13, 62, 101, or 171 μM, and measures of NAD^+ content or cell viability were made at 1, 2, 4, 8, 12, 16, 20, 24, 48, or 72 hr. NAD^+ and viability measurements were made as described in following sections.

Quality control assessments were conducted on the vehicle control data for both NAD^+ depletion and cytotoxicity data to assess outlier data points. For NAD^+ analyses, a quadratic regression model was fitted to the data using time as the independent variable. Three standard deviation limits for NAD^+ concentration data were compiled as a function of time. Average values falling outside the three standard deviation limits were considered suspect, and the vehicle control data and data from all treatment groups at that time point were eliminated prior to plotting and performing statistical analyses. For the cytotoxicity assay, the vehicle control data obtained over the first 24 hr were pooled. A mean value and three standard deviation limits were calculated. Values falling outside the three standard deviation limits were considered suspect, and the data from all the treatment groups at that time point were eliminated from subsequent statistical analyses. Data from the 48 hr and 72 hr time points were excluded from this process due to the increased frequency of low viability values observed at these times.

Statistical analyses were performed on two endpoints, the normalized responses (fractions of vehicle control) for NAD^+ concentration and cell viability. These analyses assessed the effects of compound concentration and addition mode on each endpoint. The same analyses were performed separately for each endpoint, treatment group, HD concentration, and time. All vehicle and baseline controls were excluded from analyses; no analysis was performed if either the HD concentration was zero or if the time was zero. For each endpoint, compound, HD concentration, and time point, the following analysis of variance model was fitted to the data:

$$Y_{ijk} = \mu_{ij} + \epsilon_{ijk}$$

where: Y = measured response (normalized NAD^+ concentration or cell viability),
i = compound concentration (NM: 0, 0.01, 0.1, or 1 mM; NI: 0, 0.1, 1, or 10 mM),
j = addition mode (single or multiple),
k = replicate number (1, 2, or 3),
ϵ_{ijk} = error variability for the kth replicate of the ith treatment compound concentration using the jth addition mode.

Three types of hypothesis tests were conducted using the fitted analysis of variance models:

(1) Comparisons to Treatment Control (6 Comparisons) - For both addition modes, the average observed response at each compound concentration was statistically compared to the average response of the vehicle control (0 mM NM or 0 mM NI). A positive result indicated that the average observed response for the treatment group was greater than that observed for the vehicle control, whereas a negative result indicated that the vehicle control response was greater than the treatment group response.

(2) Linear Trends Among Treatment Concentrations (2 Comparisons) - For each addition mode, a hypothesis test was conducted to determine if the treatment concentration-response relationship was statistically significant. A positive value for the linear contrast indicated an increasing relationship, whereas a negative value indicated a decreasing relationship.

(3) Comparisons Between Addition Modes (1 Comparison) - The average observed response from the multiple addition experiments was statistically compared to that from the single addition experiment. A positive value for the comparison

indicates that the average response for the multiple addition experiments was greater than that for the single addition experiments; a negative value for the comparison indicates that the reverse was true.

These nine comparisons were performed for each combination of response (normalized NAD^+ concentration or cell viability), compound (NM or NI), HD concentration (13, 62, 101, or 171 μM), and time point (1, 2, 4, 8, 12, 16, 20, 24, 48, or 72 hr).

2.6 Cellular Protein Evaluations

HEKs grown in six-well microplates were exposed to various HD concentrations when the tertiary cultures were 50 to 80 percent confluent. At 0, 2, 24, 48, and 72 hr following addition of HD, each culture supernatant was aspirated and placed into a 15 mL conical centrifuge tube. Adherent cells were detached from the plastic growth surface with trypsin-EDTA, and combined with the supernatant and 3 mL of 1 percent fetal calf serum in RPMI 1640 medium (Gibco BRL). A pellet was formed by centrifugation at 300 x g for five min. The supernatant was discarded and the sides of the tube swabbed to remove adhering supernatant. The pellet was resuspended in 500 μL of Hanks Balanced Salt Solution (HBSS) by refluxing gently. Aliquots of the cell suspension were taken for cell enumeration, for cell viability determination, and for protein measurements.

Cell number was determined using a Coulter Model ZM (Coulter Electronics; Hialeah, FL) cell counter. The lower size threshold was set at 8 μM using Coulter-sized microspheres. For protein content analysis, the cell suspension aliquot was mixed with an equal volume of HBSS containing 2 percent triton X-100. The sample was mixed well and the amount of protein determined using the BCA method (Pierce Chemical Company; Rockford, IL) with bovine serum albumin as the standard. For cell viability assessments, 100 μL of cell suspension was added to 900 μL of containing 30 μg of PI. Viability was determined by measuring the number of PI positive fluorescent cells out of a total of 2000 cells counted with a Becton Dickinson FACScan flow cytometer (San Jose, CA).

Results for each of the three experimental runs were standardized using the time zero value for each parameter. An analysis of variance was performed on the pooled data to assess

the effects of HD concentration and to estimate the components of variation for each data parameter. The analyses of variance were conducted using the mixed models analysis of variance procedure, Proc Mixed, in Statistical Analysis System (SAS; Cary, NC). Separate models were fitted to the data at each time point for each parameter. For each combination of time and data parameter, a hypothesis differences or the HD exposure groups was conducted. With this test, a significant p-value indicates that the averages of the four HD concentration groups and the control group are not statistically equivalent.

2.7 NAD^+ Assay

Culturing of cells contained in 96-well microplates was terminated at the desired time point by adding ice-cold perchloric acid to the wells such that the final perchloric acid concentration was 0.5 M. Just prior to assay, potassium hydroxide-potassium phosphate buffer was added to neutralize each sample pH and to precipitate the perchlorate anion as the potassium salt. The plates were centrifuged and the amount of NAD^+ in the supernatant was determined following MREF Method No. 1/In Vitro (See Appendix B).

2.8 Cytotoxicity Assay

Cell viability was assessed in cultures contained in 24-well microplates essentially following the method of Smith et al. (1990). After a 24-hr exposure period, each culture supernatant was transferred to a test tube containing 1 mL of RPMI 1640 with 5 percent fetal calf serum. Adherent cells were detached from the plastic growth surface using 1 mL of trypsin-EDTA solution and refluxing using a 1-mL pipette tip attached to an Eppendorf pipettor. The detached cells were added to the tube containing the previously harvested culture supernatant. To 1 mL of cell suspension, 50 μL of a 0.3 mg/mL PI solution in RPMI 1640 was added to yield a final concentration of 14 μM. After 5 min, aliquots of each supernatant-cell mixture were assayed for cell viability by measuring the PI incorporated into non-viable cells using a Becton Dickinson FACScan flow cytometer.

2.9 Poly(ADP-ribose) Polymerase Assay

Poly(ADP-ribose) polymerase (PADPRP) measurements were made in accordance with SOP MREF V-012 (See Appendix B). Cells from keratinocyte strain No. 732 were used to assess assay procedures. Cells were grown in either six-well microplates or 25 cm^2 tissue culture flasks.

Cells were detached from the plastic growth surface by trypsin treatment, pelleted by centrifugation, and the supernatant removed. Cells were aspirated through a 23-gauge hypodermic needle to aid cell separation, and resuspended in an ice-cold hypotonic solution consisting of 10 mM Tris (pH 7.8), 0.25 M sucrose, 1 mM EDTA, and 4 mM magnesium chloride. Suspensions were allowed to equilibrate to room temperature.

Thirty μL of cell suspension were added to 30 μL of a solubilization solution containing 100 mM Tris (pH 7.8), 120 mM magnesium chloride, and 1.5 percent triton X-100. This was necessary because cells in hypotonic solution alone failed to incorporate PI into the nucleus, as assessed by fluorescent microscopy; however, PI incorporation was observed when triton X-100 was added. Triton X-100 has been used in other cell systems to measure PADPRP activity in cell preparations (Leduc et al., 1988). When deoxyribonuclease type I (DNase; Sigma) was used as a positive control, approximately 8000 kunitz units of the enzyme was added to approximately 0.25 mL of solubilization solution and assayed in parallel with cell suspensions. DNase has been shown to stimulate PADPRP activity (Berger et al., 1978).

After an approximately 4-min incubation at room temperature with solubilization solution, 30 μL of tritiated-NAD solution (0.88 μCi/nmol NAD; approximately 2 μCi per determination) was added to each sample. After an approximately 2 or 5-min incubation, the reaction was terminated by adding approximately 1 mL of "stop" solution, an ice-cold, 25 percent tricarboxylic acid (TCA) solution. After approximately 45 min on ice with "stop" solution, the samples were placed on with Whatman GF/B glass fiber filters which had been prewashed with a 20 percent TCA solution. After filtration, the filters containing the samples were rinsed twice with approximately 10 mL of ice-cold 20 percent TCA solution, and then once with approximately 10 mL of 95 percent ethanol. The amount of NAD incorporated into

TCA-precipitable protein was estimated using the specific activity of the tritiated-NAD solution and the counting efficiency for tritium on the Beckman LS-7800 counter.

Preliminary studies were performed to assess whether preincubation of cells with a PADPRP inhibitor would be apparent using the cell preparation procedures described. In these studies, cells were incubated with or without 1 mM 3-aminobenzamide (3-AB) for approximately 3 hr at 37 degrees C. The supernatant was removed and the cells washed. The cells were then trypsinized and prepared as described for the PADPRP assay. The effect of 3-AB preincubation on DNase-stimulated PADPRP activity was assessed. A study was also performed to assess the effect of 1 mM 3-AB on DNase-stimulated PADPRP activity when added directly to the reaction mixture. In this study, 3-AB was added to the hypotonic solution used for the final cell resuspension.

3.0 RESULTS

3.1 HEK Growth Kinetics

Initial characterization of HEK Strain No. 2041-1 entailed examining growth kinetics through the fourth passage. Cultures were examined microscopically and the percent confluency was recorded as a function of time in culture. Subculturing was performed when cultures were approximately 80 percent confluent. As shown in Figure 1, consistent growth kinetics were observed through the fourth passage cultures. Only tertiary cultures were used in studies to provide experimental control and to prevent using senescent cells.

3.2 Concentration-Response for HD-Induced NAD^+ Depletion

HD concentration-response studies examining NAD^+ depletion at 24 hr after addition of HD were performed on five separate days with five experimental setups overall. Each exposure group consisted of a minimum of three observations. The results of these studies are shown in Figure 2. From these data, the HD NOEL, IC_{25}, IC_{50}, and IC_{75} were estimated for use in subsequent phases of this task.

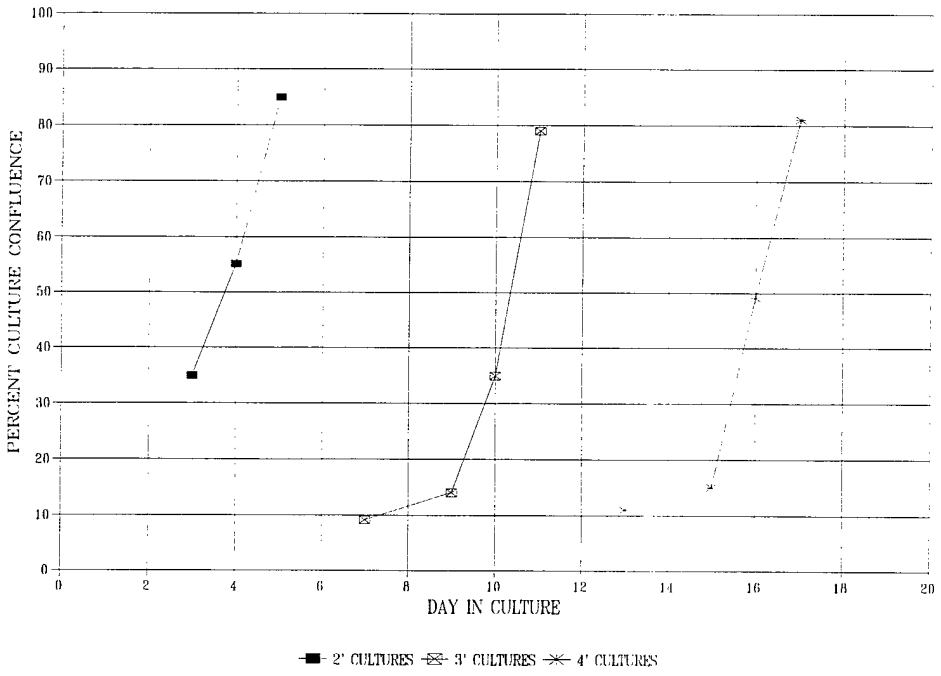

FIGURE 1. EVALUATION OF THE GROWTH KINETICS OF HUMAN EPIDERMAL KERATINOCYTE STRAIN NO. 2041-1

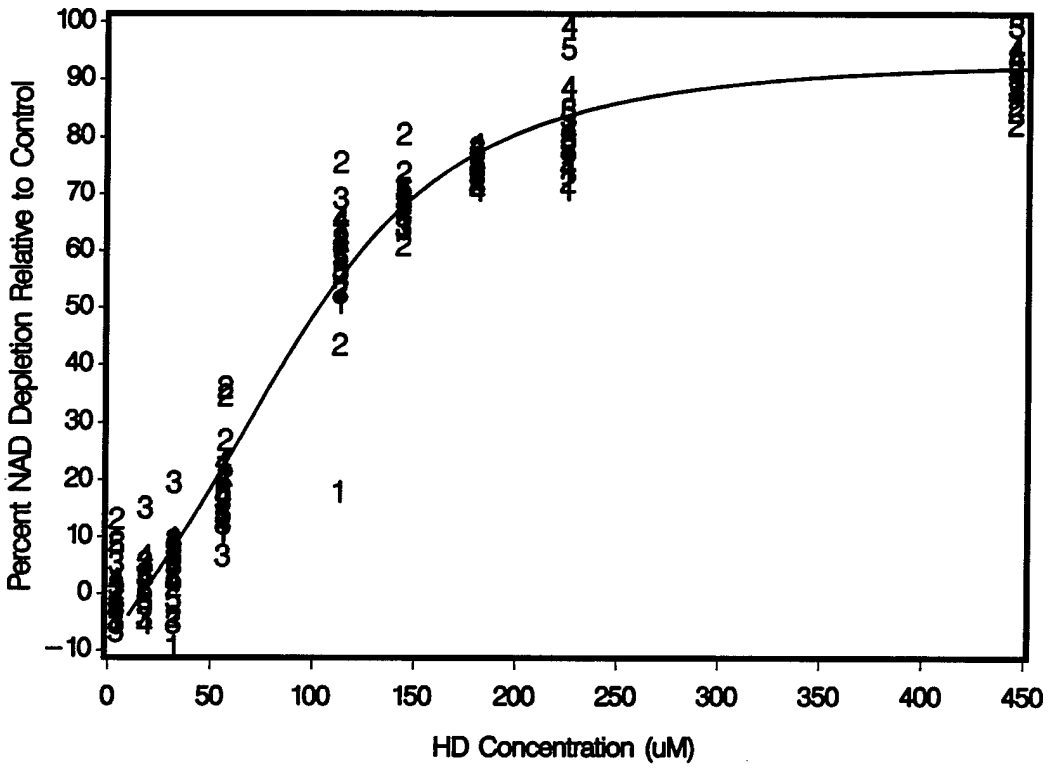

FIGURE 2. EFFECT OF HD EXPOSURE ON HUMAN EPIDERMAL KERATINOCYTE NAD^+ CONTENT 24 HR POST EXPOSURE

The HD concentrations with 95 percent confidence intervals for the various estimates are shown in Table 1. Summary statistics for these data are presented in Appendix C.

TABLE 1. SUMMARY OF PROBIT ANALYSES FOR HD-INDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DEPLETION

Parameter	n	Effective HD Concentration (μM)	
NOEL	63	13	
IC_{25} (95% C.I.)	63	62	(58, 66)
IC_{50} (95% C.I.)	63	101	(97, 105)
IC_{75} (95% C.I.)	63	171	(162, 180)
Slope (95% C.I.)		3.4	(3.0, 3.7)

3.3 Concentration-Response for HD-Induced Cytotoxicity

HD concentration-response studies assessing cell viability at 24 hr following HD addition were performed over four separate days. Each experimental run consisted of at least three measurements per HD exposure group. Data were collected on the flow cytometer for particles with a minimum diameter of 8 μM. This flow cytometer size exclusion threshold setting was confirmed using standard latex microspheres of known average diameter. The HD concentrations estimated to produce 25, 50, and 75 percent cell death at 24 hr following addition of the HD, and the slope of the concentration-cell death response curve, are presented in Table 2. Summary statistics for these data are presented in Appendix D.

TABLE 2. SUMMARY OF PROBIT ANALYSES FOR HD-INDUCED CYTOTOXICITY MEASURED BY PROPIDIUM IODIDE EXCLUSION

Parameter	n	Effective HD Concentration (μM)	
NOEL	63	29.0	
IC_{25} (95% C.I.)	63	68.5	(55.7, 78.7)
IC_{50} (95% C.I.)	63	114.0	(102, 126)
IC_{75} (95% C.I.)	63	189.0	(167, 226)
Slope (95% C.I.)		3.06	(2.33, 3.79)

3.4 Effect of NM and NI on HD-Induced NAD^+ Depletion

NAD^+ evaluations were performed on two separate days; the single and multiple addition modes of NM and NI were examined separately. Plots of the raw data and data standardized on the vehicle control value at each time point are presented in Appendix E for NM data and Appendix F for NI data. Mean values of raw and standardized data with standard deviations for each treatment group are shown in Appendix G for NM data and Appendix H for NI data. For data pooled across all HD exposure groups, NAD^+ levels were significantly ($p \leq 0.05$) decreased approximately 12 hr following addition of 101 μM HD and approximately 8 hr following addition of 171 μM HD.

Average normalized NAD^+ concentrations following 1 mM NM frequently were significantly greater than those observed for the HD-exposed controls, especially at 171 μM HD. Comparisons of average normalized NAD^+ concentrations between single and multiple addition mode experiments produced mixed results: sometimes significant protection, sometimes enhanced depletion, and sometimes no change. Since the single and multiple addition mode experiments were performed on different dates, differences in the vehicle control NAD^+ levels used for normalizing data and differences in the HD-exposed, no treatment control group responses may account for some of these results.

Average normalized NAD^+ concentrations following NI treatment frequently were significantly less than those observed for the HD-exposed, no treatment controls at all NI concentrations and at all HD concentrations. Average normalized NAD^+ concentrations were frequently greater in the multiple addition experiments than in the single addition experiments at 13 or 62 μM HD. Results were mixed at 101 and 171 μM HD.

3.5 Evaluation of NM and NI Against HD-Induced Cytotoxicity

Due to the large number of experimental treatment groups and the more labor intensive effort for the cytotoxicity assay, the experiment was limited to evaluating all NM or NI treatment groups against one or two HD concentrations. Plots of the raw data and data standardized on the vehicle control value at each time point are presented in Appendix I for NM data and Appendix J for NI data. Mean values of raw and normalized data with standard

deviations for each treatment group are shown in Appendix K for NM data and Appendix L for NI data. For data pooled across all HD exposure control groups, cell death, as measured by PI incorporation into nonviable cells, was statistically ($p \leq 0.05$) increased 16 to 24 hr following addition of 101 μM HD or 171 μM HD.

Comparisons of NM addition cultures with HD-exposed controls usually showed insignificant results at the 101 μM HD level, where toxicity was observed by 24 hr of exposure. At the 171 μM HD level, studies from the single NM addition indicated that NM provided marginal but statistically significant ($p \leq 0.05$) protection. Comparisons of NM addition modes yielded mixed results, but usually the average normalized cell viability was higher in multiple addition experiments.

Comparisons of NI treatment groups to HD-exposed controls usually showed insignificant or mixed results at the 13 and 62 μM HD concentrations. At 101 and 171 μM HD, average normalized cell viability at some timepoints was significantly greater following 1 mM NM or 10 mM NI than for the controls. Comparisons of NI addition modes yielded mixed results at all HD concentration levels.

3.6 Effect of HD on Cell Number and Protein Content

Figures 3 through 7 illustrate data parameters which have been normalized to the time zero values. Each data point is an average of three experimental runs, with each experiment consisting of a minimum of two observations. The descriptive statistics for these data are shown in Table 3. The summary statistics for each of the three runs are presented in Appendix M.

As shown in Figure 3, 13 μM HD had little initial effect on total culture protein relative to the control. The 72 hr value was significantly ($p < 0.05$) less than the respective control value. The three highest HD concentrations caused significant ($p < 0.05$) depressions in total protein which were first apparent at 24 hr following exposure. While there were increases in protein content of cultures exposed to 62 μM and 101 μM HD between 24 and 72 hr, no increase was evident in cultures exposed to 171 μM HD.

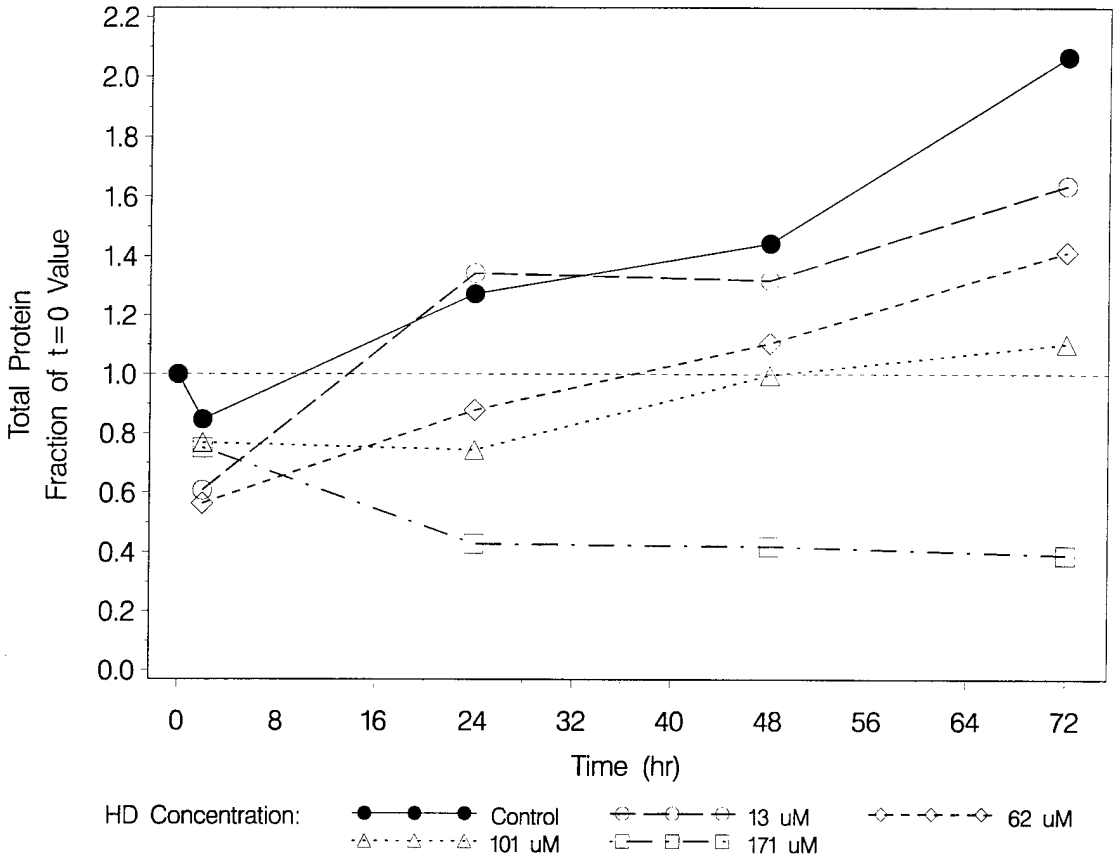

FIGURE 3. EFFECT OF HD ON TOTAL PROTEIN CONTENT OF CULTURES

TABLE 3. DESCRIPTIVE STATISTICS FOR ALL PARAMETER RESPONSES NORMALIZED TO TIME ZERO VALUES [a]

HD Conc.	Time (Hr)	N	Total Protein (μg)	Cell Count	Viability [b] PI Negative	Protein/Cell
0 μM	0	18	1.00 (0.27)	1.00 (0.25)	1.00 (0.03)	1.00 (0.25)
	2	15	0.85 (0.18)	1.10 (0.35)	0.95 (0.06)	0.83 (0.24)
	24	18	1.27 (0.36)	1.27 (0.43)	0.93 (0.07)	1.05 (0.25)
	48	17	1.44 (0.59)	1.61 (0.78)	0.76 (0.16)	0.96 (0.26)
	72	17	2.07 (0.85)	2.03 (0.77)	0.83 (0.24)	1.01 (0.20)
13 μM	2	9	0.61 (0.13)	0.89 (0.48)	0.89 (0.06)	0.85 (0.39)
	24	9	1.34 (0.30)	0.96 (0.15)	0.89 (0.08)	1.40 (0.40)
	48	9	1.32 (0.37)	0.73 (0.24)*	0.89 (0.06)*	2.28 (1.59)*
	72	8	1.64 (0.47)	0.66 (0.24)*	0.81 (0.12)	2.87 (1.66)*
62 μM	2	8	0.56 (0.39)	0.87 (0.24)	0.88 (0.16)	0.66 (0.39)
	24	8	0.88 (0.16)*	0.77 (0.24)*	0.89 (0.09)	1.23 (0.37)
	4	8	1.10 (0.36)*	0.63(0.15)*	0.89 (0.07)*	1.86 (0.77)*
	72	9	1.41 (0.33)*	0.75(0.20)*	0.88 (0.16)	1.98 (0.65)*
101 μM	2	9	0.77 (0.32)	0.88 (0.33)	0.92 (0.06)	0.88 (0.24)
	24	9	0.74 (0.18)*	0.62 (0.21)*	0.88 (0.04)	1.26 (0.39)
	48	8	1.00 (0.49)*	0.53 (0.18)*	0.87 (0.04)*	2.14 (1.27)*
	72	9	1.10 (0.15)*	0.50 (0.11)*	0.84 (0.10)	2.34 (0.87)*
171 μM	2	9	0.75 (0.30)	0.73 (0.36)*	0.90 (0.04)	1.14 (0.37)
	24	9	0.43 (0.12)*	0.59 (0.17)*	0.50 (0.29)*	0.78 (0.30)
	48	8	0.42 (0.16)*	0.74 (0.18)*	0.24 (0.19)*	0.61 (0.28)
	72	9	0.39 (0.09)*	0.58 (0.24)*	0.19 (0.24)*	0.76 (0.27)

* Response (normalized) mean was significantly different from the HD = 0 μM group mean ($p < 0.05$) for the corresponding time point.

[a] Values shown are means with standard deviations in parenthesis.

[b] Values are percent PI negative values normalized to the time zero value.

Figure 4 illustrates the effect of HD on total cell number. The three highest HD concentrations had significant ($p < 0.05$) inhibitory effects on cellular proliferation by 24 hr of exposure, while the effect of the lowest concentration was not significant ($p < 0.05$) until 48 hr. The effect of HD on cell viability is shown in Figure 5. Cytotoxicity was statistically significant in cultures exposed to 171 μM HD, but was not significant at the lower HD concentrations. This effect at the highest HD concentration was significant by 24 hr of exposure and increased with time.

Data for viable cell number, obtained by multiplying total cell number by the fraction of viable cells, are presented in Figure 6. These data are similar to the total cell count data of Figure 4 with the exception of the 171 μM HD exposed groups, which are markedly lower due to the cytotoxic effect occurring at this concentration .

Figure 7, present data for protein values upon cell number and viable cell number. The three lowest concentrations of HD increased the amount of protein per cell by 24 hr, but this was not statistically significant ($p < 0.05$) until 48 hr. The amount of protein per cell tended to increase as a function of time following HD exposure. The highest HD concentration, 171 μM, did not significantly ($p < 0.05$) affect the amount of protein per cell. Normalizing protein on a viable cell basis makes little difference in the values of the three lowest HD concentrations relative to the protein per total cell data. The values for the 171 μM HD, however, are markedly affected and indicate an increase in the amount of protein per viable cell.

If the NAD^+ data from the first set of studies (NM and NI Evaluation Studies) are normalized by the cell number and protein data obtained from subsequent studies, estimates of the amounts of NAD^+ per cell and per mg of protein can be obtained. These data, expressed on a percent of control basis (Tables 4 and 5), should be interpreted with caution since the NAD^+ values were obtained with cultures established in 96-well microplates and the cell count and protein data were obtained at a later date in cultures contained in 6-well plates. Cell numbers and protein content were estimated for the 96-well cultures using a correction factor based upon the surface area of 6-well versus 96-well plates. Using this factor, the estimated NAD^+ content is approximately 300 pmol per 10^5 cells. This value is in agreement with

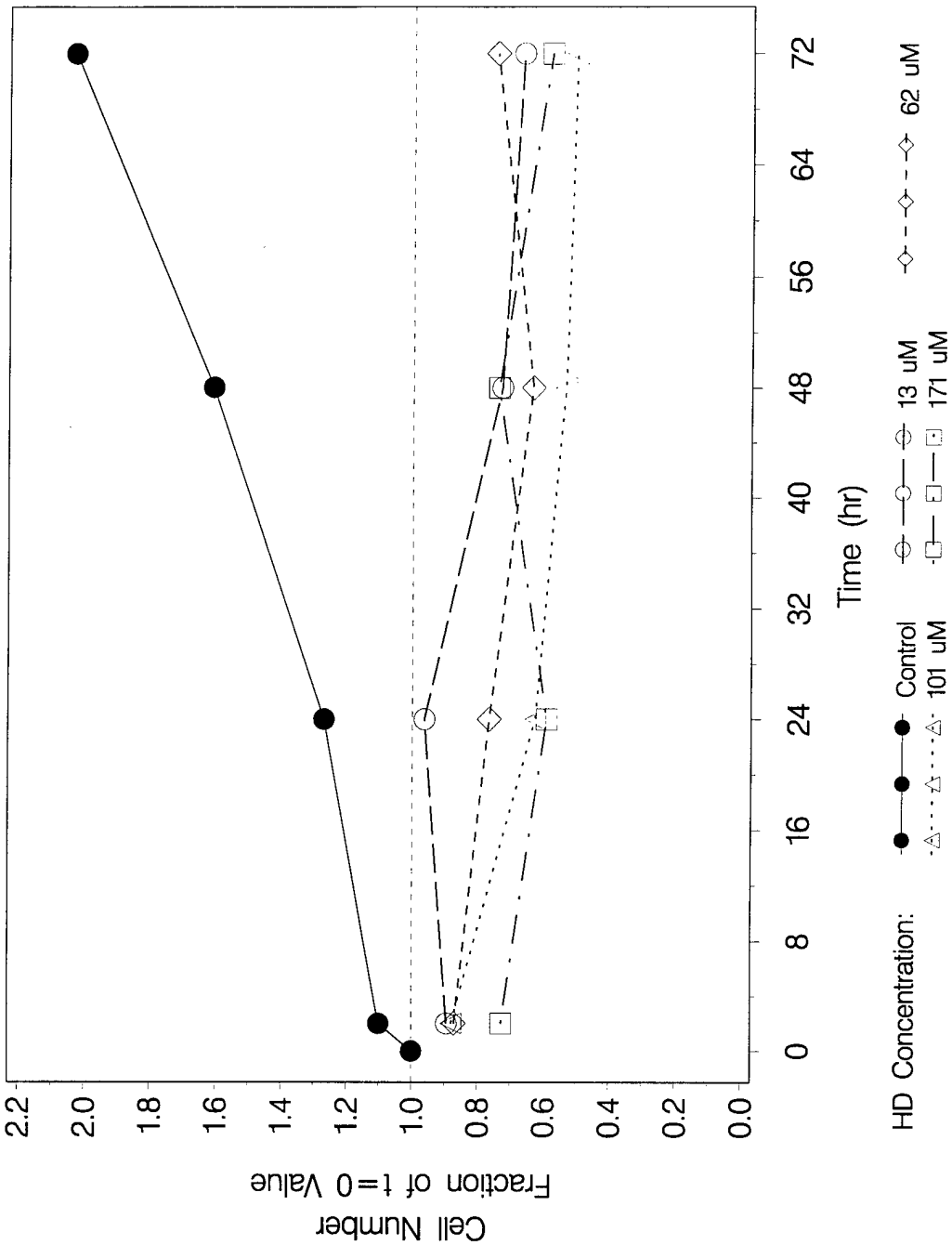

FIGURE 4. HD-INDUCED ALTERATIONS OF CELL NUMBER

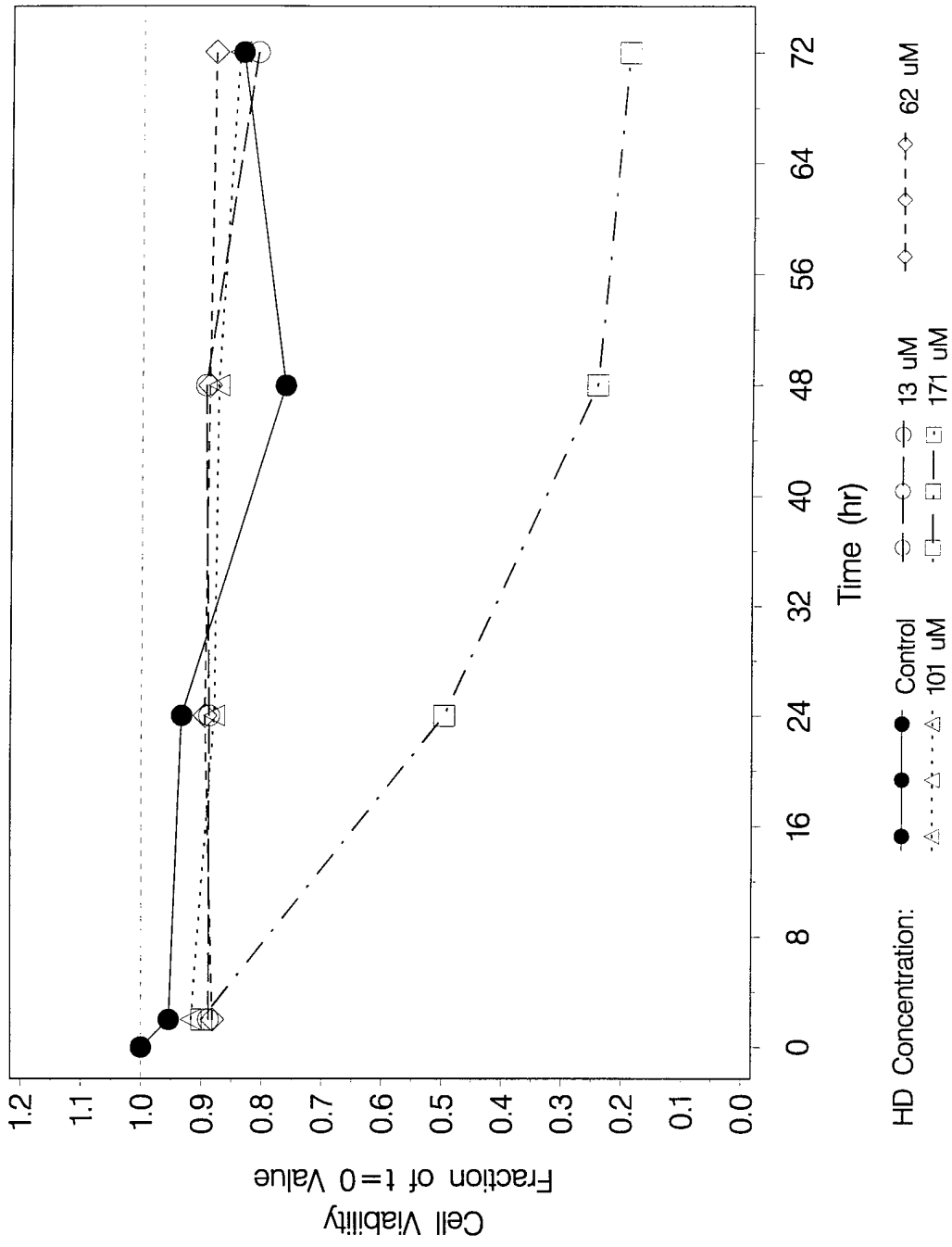

FIGURE 5. EFFECT OF HD ON CELLULAR VIABILITY MEASURED BY PROPIDIUM IODIDE EXCLUSION

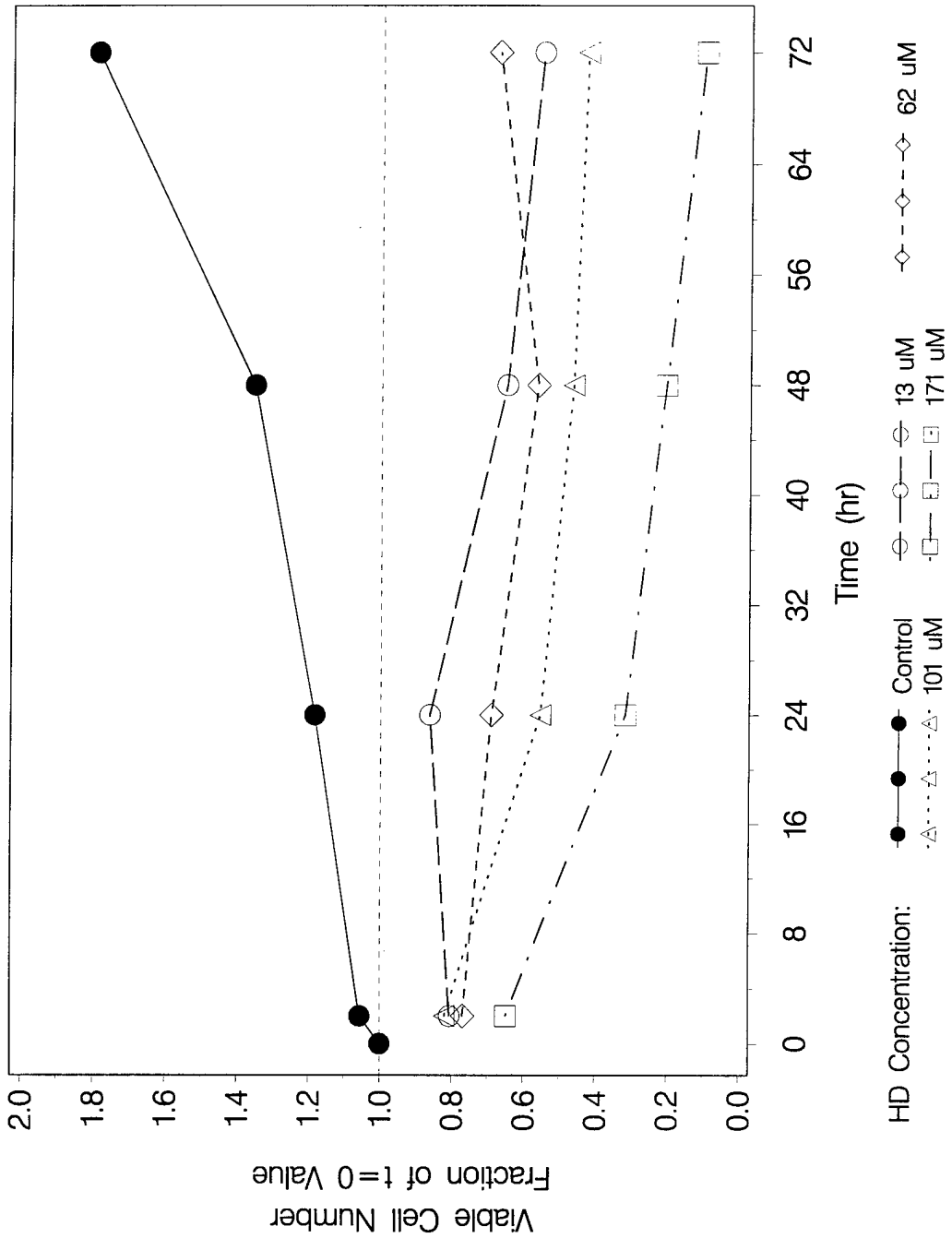

FIGURE 6. ESTIMATION OF VIABLE CELL NUMBER FOLLOWING HD EXPOSURE

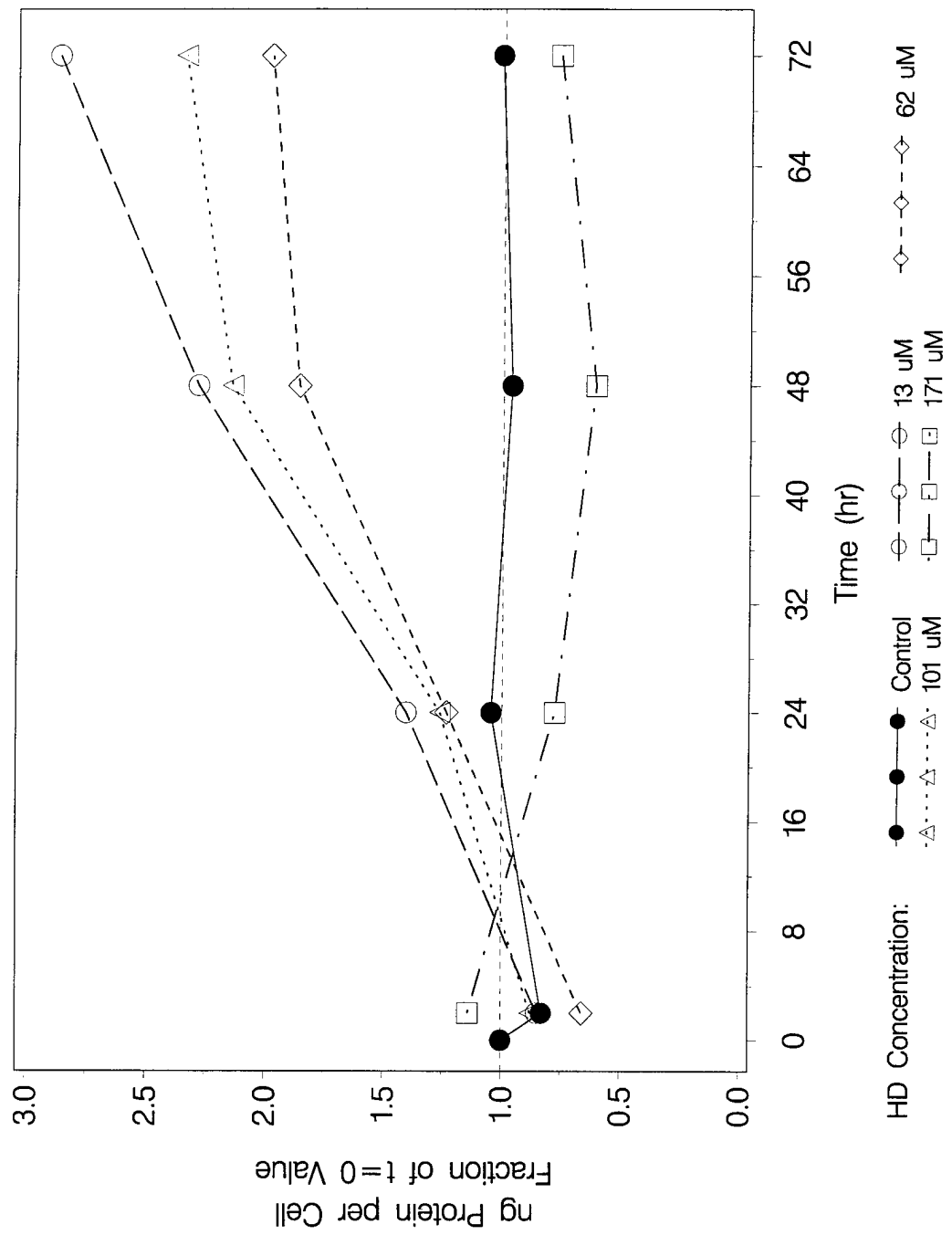

FIGURE 7. ESTIMATES OF THE AMOUNT OF PROTEIN PER CELL FOR EACH HD EXPOSURE GROUP

previously published NAD^+ values for HEK cultures (Smith et al., 1990). NAD^+ content per cell and per ug protein are elevated relative to control cultures at all HD concentrations at the 2 hr time point, and at the three lowest HD concentrations at extended time points. NAD^+ content is only decreased, relative to controls in cultures exposed to 171 μM HD. This effect appears to occur at 24 hr for NAD^+ values based on cell number, and at 48 hr for values based on protein.

TABLE 4. ESTIMATION OF NAD^+ CONTENT PER CELL AND THE EFFECT OF HD EXPOSURE*

[HD] μM	Percent of Control NAD^+ Per Cell at Respective Time Points			
	2 Hr	24 Hr	48 Hr	72 Hr
13	133	100	239	293
62	121	145	188	178
101	138	139	183	225
171	172	69	19	21

*Data are expressed as a percent of control at each time point. The NAD^+ per cell values were estimated using the initial NAD^+ data and the cell count data from protein evaluation studies.

TABLE 5. ESTIMATION OF NAD^+ CONTENT PER MICROGRAM PROTEIN AND THE EFFECT OF HD EXPOSURE*

[HD] μM	Percent of Control NAD^+ Per μg Protein at Respective Time Points			
	2 Hr	24 Hr	48 Hr	72 Hr
13	151	70	119	121
62	143	268	96	96
101	124	115	87	113
171	128	95	21	33

*Data are expressed as a percent of control at each time point. The NAD^+ per μg protein values were estimated using the initial NAD^+ data and the cell count data from protein evaluation studies.

Assuming that NM does not alter cellular protein content or cell number in HD-exposed cultures, NAD^+ values based upon cell number and protein content were estimated for NM-treated cells and are presented in Tables 6 and 7. These data suggest that 1 mM NM may be able to prevent NAD^+ depletion at 24 hr, but this effect is lost by 48 hr. The values normalized on a protein basis suggest that NM provides some level of protection at 48 hr, similar to the values based on cell number.

TABLE 6. ESTIMATION OF NAD^+ CONTENT PER CELL AND THE EFFECT OF NM ON HD EXPOSURE*

[HD] μM	[NM] mM	Percent of Control NAD^+ Per Cell at Respective Time Points			
		2 Hr	24 Hr	48 Hr	72 Hr
101	0	138	139	183	225
101	1	165	155	202	269
171	0	172	69	19	21
171	1	207	88	38	24

*Data are expressed as a percent of control at each of the time points. The NAD^+ per cell values were estimated using the initial NAD^+ data and the cell count data from the protein evaluation studies.

TABLE 7. ESTIMATION OF NAD^+ CONTENT PER MICROGRAM PROTEIN AND THE EFFECT OF NM ON HD EXPOSURE*

[HD] μM	[NM] mM	Percent of Control NAD^+ Per μg Protein at Respective Time Points			
		2 Hr	24 Hr	48 Hr	72 Hr
101	0	124	115	87	113
101	1	226	134	96	135
171	0	128	95	21	33
171	1	169	121	42	38

*Data are expressed as a percent of control at each of the time points. The NAD^+ per μg protein values were estimated using the initial NAD^+ data and the cell count data from the protein evaluation studies.

3.7 Poly(ADP-ribose) Polymerase Assay

HEK strain No. 732 was used for developing the PADPRP procedure. On the average, PADPRP activity in this strain incorporated 8.51 pmol of NAD^+ per mg protein during a five min incubation. DNase-stimulated activity to 41.3 pmol NAD^+/mg protein/5 min on average. These activity levels are similar to that reported for alkylator- stimulated PADPRP activity (20 pmol/mg/5 min) in HEKs (Rosenthal et al., 1993).

TABLE 8. EFFECT OF DEOXYRIBONUCLEASE ON POLY(ADP-RIBOSE) POLYMERASE ACTIVITY

		PADPRP* (pmol/mg/5min)		
Run No.	HEK Strain	Control	DNase	Stimulation
1	732	11.1 ± 1.52	38.2 ± 6.65	3.4
2	732	1.73 ± 1.28	34.3 ± 3.26	20.0
3	732	10.2 ± 1.21	51.4 ± 13.0	5.0
4	732	11.0 ± 4.60	N.D.	N/A

*Values are mean ± standard deviation of three observations.
N.D. - Not Determined
N.A. - Not Applicable

Studies were designed to assess the effect of NM on HD-induced cytotoxicity, NAD^+ depletion, and PADPRP activity. Since the interaction of PADPRP inhibitors such as NM and 3-AB is reversible, and cellular preparation for the PADPRP assay requires removal of the supernatant containing the inhibitors, experiments were performed to assess the effect of cellular preparation on 3-AB inhibition of DNase-stimulated PADPRP activity. When 1 mM 3-AB is included in the reaction mixture, DNase-stimulated PADPRP activity is completely inhibited (Table 9).

TABLE 9. EFFECT OF 3-AMINOBENZAMIDE (3-AB) ON DEOXYRIBONUCLEASE (DNase) STIMULATED POLY(ADP-RIBOSE) POLYMERASE ACTIVITY

Group	PADPRP Activity pmol/mg/5 min
Control	1.73 ± 1.28
DNase	34.3 ± 3.26
3-AB*	0.35 ± 0.36

*3-Aminobenzamide (1 mM final) was included in the reaction mixture for the PADPRP determination. These data are from the second run as shown in Table 8.

When cells were preincubated with 1 mM 3-AB for a period of time sufficient to allow equilibration across cell membranes and the supernatant was removed in preparation for PADPRP determination, the majority of the DNase-stimulated activity was still inhibited (Table 10). The raw data for the PADPRP measurements is present in Appendix N.

TABLE 10. EFFECT OF CELLULAR INCUBATION WITH 3-AMINOBENZAMIDE (3-AB) ON DEOXYRIBONUCLEASE (DNase) STIMULATED POLY(ADP-RIBOSE) POLYMERASE ACTIVITY

Group	PADPRP Activity pmol/mg/5 min
Control	10.2 ± 1.21
DNase	51.4 ± 13.0
3-AB*	18.1 ± 7.73

*3-Aminobenzamide (1 mM final) was added to cultures 3 hr prior to harvesting for PADPRP determination. 3-AB was not included in the reaction mixture during the PADPRP determination. Data are from the third experimental run shown in Table 8.

As the assay was scaled up to accommodate more samples and HD-exposed cells were utilized, problems with the assay were encountered. These problems seemed to stem from elevated background counts. Preliminary studies were conducted in which the assay was performed in a Millipore Multiscreen format. This procedure showed promise, including an approximately 10-fold reduction in liquid radioactive waste, however these evaluations were not completed.

4.0 DISCUSSION

The hypothesis that DNA-damaging agents cause PADPRP activation, NAD^+ depletion, metabolic shutdown, and cell death was originally proposed by Berger in 1980. A similar theory which also included protease release during cell death was postulated as the mechanism for HD-induced vesication (Papirmeister et al., 1985). Support for this hypothesis comes primarily from *in vitro* data which demonstrates HD-induced PADPRP activation and NAD^+ depletion (Meier et al., 1987; Smith et al., 1990). Additional support comes from the use of PADPRP inhibitors to prevent HD-induced NAD^+ depletion and cytotoxicity in human lymphocytes (Meier et al., 1992; Smith et al., 1988). Similar types of studies have been performed with HEK cultures, but the results have not been consistent. While NM, the PADPRP inhibitor and NAD^+ precursor, has been shown to prevent HD-induced NAD^+ depletion (Mol et al., 1989; Smith et al., 1990; Joiner et al., 1990; Martens, 1992), the results of studies that measure cytotoxicity by PI exclusion and glucose utilization are incongruous. One mM NM was found to provide only marginal protection against HD-induced cell death as measured by PI exclusion in this study. These data are consistent with the data of Mol et al. (1989), who did not observe a significant effect of NM on HEK viability as measured by glucose utilization, and the data of Martens (1993) who saw only a marginal effect of NM on HD-induced cytotoxicity in the PI exclusion assay.

Initial *in vivo* studies with hairless guinea pigs indicated that NM was able to provide protection against HD-induced microvesication (Yourick et al., 1992). Data from the isolated perfused porcine skin flap model indicated that NM may provide some protection from HD-induced basal cell toxicity, but was unable to prevent HD-induced microblister formation

(Zhang et al., 1995). Likewise, NM was able to prevent HD-induced reduction in glucose utilization by human skin *ex vivo*, but NM did not affect HD-induced microblister formation (Mol et al., 1991). These data indicate that NM may provide some protection against HD-induced cytotoxicity, but not prevent HD-induced microvesication. These data also indicate that basal cell toxicity may not be necessary for microvesication.

DNA alkylation by HD inhibits proliferation by blocking the cell cycle at the G1-S and G2-M boundaries at vesicating doses (>50 μM). The G1-S block is caused by approximately 10-fold higher HD concentrations then the G2-M block (Smith et al., 1993). The effect of HD exposure on cellular protein levels was examined to assess the usefulness of protein as a measure for normalizing biochemical measures. All HD concentrations inhibited cellular proliferation, however cells exposed to the three lowest HD concentrations (13, 62, and 101 μM) did not exhibit toxicity and had elevated levels of protein per cell relative to control cells. This effect was significant by 48 hr and was highest (2.3-fold $>$ controls) at the 13 μM HD exposure level, and somewhat lower at the 62 and 101 μM HD exposure levels (1.9 and 2.1 times that of controls, respectively). This is consistent with the cell cycle data of Smith et al. (1993), which indicated that a G2-M cell cycle block should be occurring at HD levels in this concentration range. This is also consistent with the finding of cellular enlargement with continued RNA and protein synthesis with alkylating agent-induced G2-M cell cycle blocks (Calabresi and Chabner, 1990).

In contrast, the amount of protein per cell at the 171 μM HD concentration was not significantly different than controls. At this HD concentration there was no apparent recovery of protein synthesis, and cytotoxicity was observed within 24 hr of exposure. Results at this exposure level are markedly different than those for the 101 μM HD group. Data from Smith et al. (1993) indicate that this exposure level may be consistent with a G1-S block and would be considered at the vesicating level. These data are consistent with the findings of Mol and deVries-VandeRuit in which HD concentrations in the vesicating range inhibited DNA replication and also inhibited protein synthesis. These data are also interesting in respect to previous findings of Martens and Smith that indicated a shift in the cellular energy source away from glucose at HD concentrations starting at approximately 70 μM. The data of

Martens and Smith, indicate that HD exposures of 70 μM or greater cause the ratio of glucose utilization to lactate production to increase above two, indicating that a carbon source other than glucose is being used to produce lactate. It is possible that protein synthesis or other anabolic processes involved in cellular maintenance and function are being sacrificed, or enhanced catabolism of proteins, lipids, or other sources is occurring in the effort of a cell to sustain energy supplies.

Even though protein was not found to be predictive of cell number in studies conducted under this task, the literature does indicate that protein may be predictive of the increased cell volume that occurs with asynchronous cell growth (Cohen and Studzinski, 1967). With the compartmentalization of cofactors (e.g., NAD^+), proteins, etc., being critical for cellular function, it is not completely clear whether cell number or cellular volume is more appropriate for biochemical measurement normalization. In this respect, the NAD^+ data from earlier studies were normalized both on average cell number and cell protein values obtained from studies conducted at a later point in this task. When the NAD^+ data in this report was standardized on a cell basis, then cellular NAD^+ levels were somewhat depressed at the same concentration of HD required to produce cytotoxicity. If the data were normalized on a protein content basis, however, then NAD^+ depression was not observed until after cytotoxicity had occurred. Assuming that NM does not affect protein levels, NM normalization using protein content and cell number data for control or HD-exposed cultures would indicate that NM provides some protection from HD-induced NAD^+ depletion at 24 and 48 hr following exposure.

5.0 CONCLUSIONS

Results from these studies provide evidence that NM, at the concentrations evaluated, would be an effective pretreatment for HD-induced microvesication. One mM NM provided only marginal protection against HD-induced HEK cytotoxicity. It is possible that PADPRP inhibitors that are more potent than NM may prove to have efficacy against HD-induced cytotoxicity and microvesication.

These studies indicate that HD concentrations as low as 13 μM inhibit HEK reproduction. When making biochemical measurements on HD-exposed cultures at extended time points, the anti-proliferative effect of HD needs to be considered. Protein content does not appear to be a viable alternative to cell number for normalization, but may be predictive of cellular volume as suggested by Cohen and Studzinski. It may be worthwhile to assess the relationship between cellular protein and cellular volume in HD-exposed cells. Cell cycle or cellular proliferation assays, which would take into account cell number or number of colonies as well as viability, may prove useful for evaluating therapeutic regimens designed to overcome the cell cycle blocks induced by HD.

The protein content, cell number, and cell viability data produced in this project are interesting in that marked differences exist in cellular protein levels and viability between 101 μM and the 171 μM HD exposure groups. These differences seem to occur in the HD concentration range reported to cause vesication (50 to 100 μM or higher). If cessation of protein synthesis is an event associated with vesicating doses of HD as suggested by Mol and deVries-VandeRuit, then the biochemical events leading to this shutdown may provide insight into the development of a new therapeutic strategy to minimize HD-induced dermal toxicity.

6.0 ACKNOWLEDGMENTS

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7.0 REFERENCES

Berger, N.A., G.W. Sikorski, S.J. Petzold, and K.K. Kurohara. (1979). "Association of Poly(Adenosine Dephosphoribose) Synthesis with DNA Damage and Repair in Normal Human Lymphocytes". *J. Clin. Invest.* 63:1164-1171.

Berger, N.A., G. Weber, A.S. Kaichi, and S.J. Petzold. (1978). "Relation of Poly (Adenosine Diphosphoribose) Synthesis to DNA Synthesis and Cell Growth". *Bioch. Biophys. Acta* 509:105-117.

Calabresi, P. and B.A. Chabner. (1990). Chemotherapy of Neoplastic Diseases. in "Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition". A. Gilman, T. Rall, A. Nies, and P. Taylor, eds. Pergamon Press, New York.

Cappizzi, R.L., W.J. Smith, R.J. Field, and B. Papirmeister. (1973). "A Host-Mediated Assay for Chemical Mutagen using the L5178Y/asn(-) Murine Leukemia". *Mutat. Res.* 21:6.

Carson, D.A., S. Seto, D.B. Wasson, and C.J. Carrera. (1986). "DNA Strand Breaks, NAD Metabolism, and Programmed Cell Death". *Exp. Cell Res.* 164:273-281.

Cohen, L.S. and G.P. Studzinski. (1967). "Correlation Between Cell Enlargement and Nucleic Acid and Protein Content of HeLa Cells in Unbalanced Growth Produced by Inhibitors of DNA Synthesis". *J. Cell Physiol.* 69:331-340.

Gross, C.L., H.L. Meier, B. Papirmeister, F.B. Brinkley, and J.B. Johnson. (1985). "Sulfur Mustard Lowers Nicotinamide Adenine Dinucleotide Concentrations in Human Skin Grafted to Athymic Nude Mice". *Toxicol. App. Pharmacol.* 81:85-90.

Jacobson, E.L. and M.K. Jacobson. (1989). "Pyridine Nucleotide Levels as a Function of Growth in Normal and Transformed 3T3 Cells". *Arch. Bioch. Biophys.* 175:627.

Joiner, R.L. , G.S. Dill, D.W. Hobson, and J.A. Blank. (1990). Final Report for MREF Task Order No. 88-36 entitled "Development of In Vitro Screening Assays for Candidate Pretreatment and Treatment Compounds", Performed for USAMRICD under Contract DAMD17-83-C-3129.

Leduc, Y., J.J. Lawrence, G. DeMurcia, and G.G. Poirer. (1988). "Cell Cycle Regulation of Poly(ADP-Ribose) Synthetase in FR3T3 Cells". *Bioch. Biophys. Acta* 968:275-282.

Martens, M.E. (1992). "Biochemical Studies of Sulfur Mustard-Induced Injury". *Proc 4th Symposium on Protection Against Chemical Warfare Agents*. 325-331.

Martens, M.E. and W.J. Smith. (1993). "Mechanisms of Sulfur Mustard-Induced Metabolic Injury". *Med. Defense Biosci. Rev.*257-264.

Meier, H.L., C.L. Gross, L.M. Graham, C.T. Lusco, and J.B. Johnson. (1987). "The Prevention of 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard, HD) Cytotoxicity in Human Lymphocytes by Inhibitors of Poly(ADP-Ribose) Polymerase". *Med. Defense Biosci. Rev.* 313-316.

Meier, H.L. and J.B. Johnson. (1992). "The Determination and Prevention of Cytotoxic Effects Induced in Human Lymphocytes by the Alkylating Agent 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard, HD)". *Toxicol. App. Pharmacol.* 113:234-239.

Mol, M.A.E., A.B.C. VandeRuit, and A.W. Kluivers. (1989). "NAD^+ Levels and Glucose Uptake of Cultured Human Epidermal Cells Exposed to Sulfur Mustard". *Toxicol. App. Pharmacol.* 98:159-165.

Mol, M.A.E., R. DeVries, and A.W. Kluivers. (1991). "Effects of Nicotinamide on Biochemical Changes and Microblistering Induced by Sulfur Mustard in Human Skin Organ Cultures". *Toxicol. App. Pharmacol.* 107:439-449.

Mol, M.A.E., and A.B.C. deVries-VandeRuit. (1992). "Concentration and Time-Related Effects of Sulphur Mustard on Human Epidermal Keratinocyte Function". *Toxicol. In Vitro* 6:235-251.

Papirmeister, B., C.L. Gross, H.L. Meier, J.P. Petrali, and J.B. Johnson. (1985). "Molecular Basis for Mustard-Induced Vesication". *Fund. App. Toxicol.* 5:S134-S139.

Papirmeister, B. (1994). "Does Apoptosis (Programmed Cell Death) Play a Role in Sulfur Mustard Injury?" *Med. Chem. Defense* 7:1-13.

Roberts, J.J., T.P. Brent, and A.R. Crathorn. (1971). "Evidence for the Inactivation and Repair of the Mammalian DNA Template after Alkylation by Mustard Gas and Half Mustard Gas". *Eur. J. Canc.* 7:515-524.

Rosenthal, D., V. Kang, J. Ntambi, and M. Smulson. (1993). "The Expression of Antisense Transcripts to PADPRPRP and HD-Induced Preferential DNA Repair and Differentiation". *Med. Defense Biosci. Rev.* 207-216.

Ross, D.W. (1983). "Unbalanced Cell Growth and Increased Protein Synthesis Induced by Chemotherapeutic Agents". *Blood Cell* 9:57-68.

Rozmiarek, J., R.L. Cappizzi, B. Papirmeister, W.H. Furman, and W.J. Smith. (1973). "Mutagenic Activity in Somatic and Germ Cells Following Chronic Inhalation of Sulfur Mustard". *Mutat. Res.* 21:13.

Sims, J.L., G.W. Sikorski, D.M. Catino, S.J. Berger, and N.A. Berger. (1982). "Poly(adenosinediphosphoribose) Polymerase Inhibitors Stimulate Unscheduled Deoxyribonucleic Acid Synthesis in Normal Human Lymphocytes". *Biochem.* 21:1813-1821.

Smith, W.J., K.M. Sanders, C.L. Gross, and H.L. Meier. (1988). "Flow Cytometric Assessment of the Effects of Alkylation on Viability, Cell Cycle, and DNA Structure in Human Lymphocytes". *Cytometry Supp.* 2:21.

Smith, W.J., C.L. Gross, P. Chan, and H.L. Meier. (1990). "The Use of Human Epidermal Keratinocytes in Culture as a Model for Studying the Biochemical Mechanisms of Sulfur Mustard Toxicity". *Cell Biol. And Toxicol.* 6:285-291. (1990).

Smith, W.J., K.M. Sanders, J.E. Caulfred, and C.L. Gross. (1991). "Sulfur Mustard-Induced Alterations of DNA Structure and Kinetics in Proliferating Human Cells in Culture". *Med. Defense Biosc. Rev.* 105-108.

Smith, W.J., K.M. Sanders, S.E. Ruddle, and C.L. Gross. (1993). "Cytometric Analysis of DNA Changes Induced by Sulfur Mustard". *Med. Defense Biosci. Rev.* 189-198.

Yourick, J.J., C.R. Clark, and L.W. Mitcheltree. (1991). "Niacinamide Pretreatment Reduces Microvesicle Formation in Hairless Guinea Pigs Cutaneously Exposed to Sulfur Mustard". *Fund. App. Toxicol.* 17:533.

Yourick, J.J., J.S. Dawson, and L.W. Mitcheltree. (1992). "Sulfur Mustard-Induced Microvesication in Hairless Guinea Pigs: Effect of Short-Term Niacinamide Administration". *Toxicol. App. Pharmacol.* 117:104-109.

Zhang, Z., J.E. Riviere, N.A. Monteiro-Riviere. (1995). "Evaluation of Protective Effects of Sodium Thiosulfate, Cysteine, Niacinamide, and Indomethacin on Sulfur Mustard-Treated Isolated Perfused Porcine Skin". *Chemico-Biol. Interact.* 96:249-262.

APPENDIX A

PROTOCOL 82

Time- and Dose-Response Characterization of the Prevention of HD-Induced NAD+ Depletion and HD-Induced Cytotoxicity by Niacinamide and Niacin

Study performed by Battelle Memorial Institute
505 King Avenue, Columbus, Ohio 43201-2693

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2. Study Director: James A. Blank, Ph.D.

3. Study Supervisor: Rebekah A. Starner, B.S.

4. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

5. Sponsor Monitor: LTC Don W. Korte, Jr., Ph.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

6. Background: A research objective of primary importance for the U.S. Army Medical Chemical Defense Research program is to characterize the pathophysiology produced by sulfur mustard (HD) in order to design effective therapeutic interventions. One current hypothesis to explain HD pathophysiology is centered on the poly(ADP-ribose) polymerase (PADPRP) enzyme which is activated by HD-induced deoxyribonucleic acid (DNA) damage. Once activated, PADPRP rapidly depletes cellular nicotinamide adenine dinucleotide (NAD^+) which is hypothesized to result in cell death and to initiate vesication. Studies testing this hypothesis have used different cell culture systems, different concentrations of HD, and different times of therapeutic compound intervention. Therapeutic compounds include, at present, precursors for NAD^+ synthesis (i.e., niacin and niacinamide (NM)) and also inhibitors of PADPRP activity (i.e., NM). Recent studies have shown that NM can provide limited protection of normal human epidermal keratinocytes (HEK) in vitro at 24-hr, but not 72-hr post exposure. Characterization of the time- and concentration-response relationships between HD and NM or niacin in a widely used experimental cell culture system are needed to more completely define relationships between NAD^+ depletion, PADPRP stimulation, and cell viability crucial to the scientific evaluation of the hypothesis.

7. Objective: Task 91-22 "Time- and Dose-Response Characterization of the Prevention of HD-Induced NAD^+ Depletion and HD-Induced Cytotoxicity by Niacinamide and Niacin" is involved with examining the time dependent relationships between NM and niacin concentration and HD-induced NAD^+ depletion, PADPRP activation, and cytotoxicity using a normal HEK cell system. These studies should provide valuable information regarding

the role of NAD^+ depletion and PADPRP activation in HD-induced cellular pathology and provide valuable information regarding the effectiveness of NM against HD-induced toxicity.

8. Experimental Design:

A. Cell Culture Conditions - Tertiary cultures of HEKs are used for these studies. Secondary HEK cultures are harvested and seeded into test plates at a density of 40,000 to 50,000 cells per square centimeter. Cultures between 60 to 80 percent confluent, as determined by low power microscopic inspection, are used for these studies. Incubations and culture conditions are fixed at 37 C (± 5 C), 5 percent carbon dioxide (± 0.5 percent), and saturated humidity atmosphere for all studies.

B. HD Concentrations Response Studies - Concentration response studies are performed to establish the HD concentrations that produce no observable effect (NOEL) on NAD^+ depletion, deplete cellular NAD^+ levels by 25 percent (IC_{25}), 50 percent (IC_{50}), 75 percent (IC_{75}) of control at 24 hr post HD exposure. The HD NOEL, IC_{25}, IC_{50}, and IC_{75} for cytotoxicity are also determined.

1. NAD^+ Assay - Tertiary cultures of HEKs are incubated in tissue culture medium with various HD concentrations for 24 hr then analyzed for cellular NAD^+ depletion following the procedures described in Section 8.E.1. Each experiment consists of at least eight HD concentration groups with three observations per group. The experiment is repeated a minimum of three times. The established NOEL, IC_{25}, IC_{50}, and IC_{75} will then be used for the NM and niacin pretreatment studies.

2. Cellular Viability - Tertiary cultures of HEKs are incubated with various HD concentrations for 24 hr. The cell culture supernatant is transferred to another fluid vessel and trypsin solution is added to the incubation plate to detach the adherent HEKs. The detached cells are combined with the supernatant, then trypsin neutralizing solution and propidium iodide (PI) are added. Cellular viability is assessed by flow cytometry measuring PI incorporation into non-viable cells using the procedures described in Section 8.E.2. Each experiment consists of at least eight HD concentration groups with three observations per group. The experiment is repeated a minimum of three times.

The total minimum number of experimental observations to be obtained in these studies will be approximately 72 for each of the two endpoints as shown below:

TOTAL NUMBER OF ESTIMATED EXPERIMENTAL VALUES FOR HD CONCENTRATION RESPONSE STUDIES

Study Type	Minimum Number of Treatments	Number of Observations Per Treatment	Total Number of Experiments	Total Estimated Experimental Values
Cytotoxicity	8	3	3	72
NAD^+ Depletion	8	3	3	72

C. NM and Niacin Pretreatment Studies - Time Course studies are performed in the presence and absence of NM and niacin with four HD concentrations established from studies performed under Section 8.B.1. The HD concentrations are the NOEL, IC_{25}, IC_{50}, and IC_{75} for NAD^+ depletion at 24-hr post exposure. The impact of three niacin and NM concentrations on HD-induced alteration of HEK NAD^+ content, viability, and PADPRP activity is examined at 0, 1, 2, 4, 8, 12, 16, 20, 24, 48, and 72 hr for each HD concentration. All treatment groups will consist of triplicate samples.

1. Comparison of Pretreatment Methods - It has not been established whether repeated NM or niacin additions, compared to one pre-exposure addition would provide extended protection from HD-induced NAD^+ depletion, cytotoxicity, and PADPRP activation. Therefore, two pretreatment compound addition methods are evaluated for each endpoint prior to initiating the pretreatment studies.

   a. The first method of pretreatment addition is to analyze the effect of HD on cellular NAD^+ levels, viability, and PADPRP activity when NM or niacin are added 30 min prior to HD addition.

   b. The second method is to analyze the effect of repeated NM and niacin additions. In this study, pretreatment compounds are added 30 min prior to HD as well as 4, 24, and 48 hr following HD addition. Because of possible cellular detachment during culture, a technical procedure will be developed in which tissue culture supernatant can be removed and replaced with fresh medium at the indicated time points without removing the detached cells. For this purpose, a centrifugation step with removal of half the culture volume followed by adding fresh media containing pretreatment compound will be examined.

2. Pretreatment Studies - The method of choice for the NM and niacin addition is determined in conjunction with the USAMRICD Technical Point of Contact (POC), Task Area Manager (TAM), and Contracting Officer's Representative (COR) and will be used for all endpoints examined. These studies will be performed two times, providing a total of four experimental replicates when the studies in Section 8.C.1. are considered.

   The total minimum number of experimental observations to be obtained in these studies (Section 8.C.1. and 8.C.2) will be approximately 5,280 for each of the three endpoints as shown in the following table.

TOTAL NUMBER OF ESTIMATED EXPERIMENTAL VALUES FOR NIACINAMIDE AND NIACIN PRETREATMENT STUDIES

Study Type	Number of HD Groups	Number of Niacinamide Groups	Number of Niacin Groups	Number of Timepoints	Number of Observations Per Experimental Group	Total Number of Experiments[a]	Total Estimated Experimental Values
Cytotoxicity	5	4	0	11	3	4	2,640
Cytotoxicity	5	0	4	11	3	4	2,640
						Total	5,280
NAD^+ Depletion	5	4	0	11	3	4	2,640
NAD^+ Depletion	5	0	4	11	3	4	2,640
						Total	5,280
PADPRP Activation	5	4	0	11	3	4	2,640
PADPRP Activation	5	0	4	11	3	4	2,640
						Total	5,280

[a]Includes the experiments used to assess the treatment addition methods.

D. HD Additions - Exempt levels of HD will be diluted with tissue culture medium and added to cultures following the procedures set forth in Battelle SOP MREF I-003. The test plates will be set in the hood for 1 hr following HD addition to allow for HD hydrolysis and escape of any HD vapors that may form. The plates will then be transferred to an incubator. The hr in which the test plates are set in the biological safety cabinet will be considered part of the HD incubation period.

E. Endpoint Measurement - Assays may be run simultaneously, however, separate keratinocyte cultures are needed for each endpoint.

1. NAD^+ Assay - The microtest plates are centrifuged and the tissue culture supernatant is removed. Dilute perchloric acid is used to digest the cells and to release intracellular NAD^+. NAD^+ will be measured following the methodology contained in Battelle SOP MREF V-003 and MREF Method/In Vitro No. 1.

2. Cellular Viability - Battelle SOP MREF V-011 and MREF Method/In Vitro No. 15 will be followed for measuring cellular viability as applicable. The current MREF SOPs and methods are for examining human mononuclear leukocytes (a non-adherent cell type), and therefore a new MREF method or modification of an existing MREF method based upon current SOPs and USAMRICD methodology will be required for this adherent cell type. Procedures used by USAMRICD investigators for HEK cells are performed in 24-well plates and attempts will be made to adopt this assay to be performed in 96-well plates.

3. PADPRP Activation - PADPRP can modify proteins by attaching the ADP-ribose moiety of NAD^+ to the protein. PADPRP activity is measured following methodology that will be developed during the course of this task by USAMRICD and Battelle investigators. Current USAMRICD procedures use radiolabeled NAD^+ and measurement of radiolabeled proteins is assessed. To avoid the use of radioactive material, an initial attempt will be made to use a fluorescent-based assay. In order to make the assay more economical, an attempt will be made to adapt this assay to a 24-well plate system and, if possible, to a 96-well microplate system. This will result in the preparation of a new MREF method and/or SOP.

F. Assay Controls - The HD-exposed group and NM-pretreated HD-exposed group serves as the negative and positive assay controls, respectively, for all three endpoints. The assay controls are analyzed at 24 hr post HD exposure for the NAD^+ and cellular viability assays. The analysis time for the PADPRP assay controls will depend on the results of the time course studies. Chemical analyses on the stock HD vial are performed to monitor HD stock stability.

G. Historical Database - Historical databases are established and continually compiled for assay control values (negative and positive). If the mean value of positive assay control falls outside three standard deviations (STDs) of the mean historical

value, then the data are considered suspect. The mean value for the negative assay control is then compared to its historical value and if this value is not within three STDs of the mean, then experimental procedures will be reviewed to determine the cause of the extreme negative control value. If a shift in the negative control results from a new batch of keratinocytes, then the historical average will be recomputed. If the cause of the extreme value cannot be determined, then the influence of this experiment on the final results will be assessed before including or omitting the dataset.

If either the positive or negative control value falls within three STDs of their respective historical value, then the assay is considered valid and statistical comparisons on the experimental datasets are performed. Positive and/or negative assay control values falling within three STDs range defined by their respective historical databases, are then added to their respective historical databases and flagged as to whether or not they will limit the control limits. Datasets will be omitted from computing future control limits only if a cause for the extreme value can be identified. As a number of HEK strains will be used for these studies, the control values may shift. The database will be examined for response shifts and if this occurs the fixed challenge concentrations may be re-established and new database compiled.

H. Statistical Evaluations

1. HD Concentration Response Studies - Statistical models are fitted to the HD concentration response data for NAD^+ depletion and cytotoxicity to estimate the HD IC_{25}, IC_{50}, and IC_{75} for each parameter examined. Models considered may include standard parametric concentration-response curves with corrections for either natural background levels or limiting responses less than 100 percent. If parametric models are not appropriate for fitting the data then piece-wise linear regression models may be used to estimate the IC_{25}s, IC_{50}s, and IC_{75}s for one or both of the parameters.

2. Pretreatment Studies - Average levels of HD-induced NAD^+ depletion, cellular viability, and PADPRP inhibition will be plotted against time for each concentration of NM or niacin at each concentration of HD. For each response, separate analyses of variance models may be used to assess the effects of HD concentration and treatment concentration (NM or niacin) at each of the

sampling time points. If appropriate, analyses of variance with repeated measures on time may be carried out to assess the effects of HD concentration and treatment concentration (NM or niacin) over the duration of the experiment.

9. Record Maintenance: The following records are to be maintained for Task 91-22:

   A. CSM accountability log and inventory.

   B. Preparation of reagents.

   C. Decontamination and disposal records.

   D. Any other records needed to reconstruct the study and to demonstrate adherence to the protocol.

   E. All records are maintained in the Battelle archives.

10. Data Reports:

   A. An interim letter report presenting data from pretreatment addition studies for NM and niacin will be prepared and submitted.

   B. At the end of Task 91-22, a Draft Final Report is written and submitted to USAMRICD within 60 working days of completion of the task. The Draft Final Report includes, at a minimum, the following sections:

      1. Signature page of key study personnel and their responsibilities,

      2. Experimental design,

      3. Test material description and preparations,

      4. Tabulation and statistical analysis of data,

      5. Discussion and conclusion.

      Following receipt of the Draft Final Report comments from USAMRICD, the Final Report will be prepared within 28 working days.

11. Approval Signatures:

Carl T. Ols — 8/19/92

David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager — Date

James A. Blank — 19 August 1992

James A. Blank, Ph.D.
Study Director — Date

Rebekah A. Starner — 19 August 1992

Rebekah A. Starner, B.S.
Study Supervisor — Date

Don W. Korte, Jr. LTC, MS — 20 AUGUST 92

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR — Date

APPENDIX B

SOPs

METHOD FOR CULTURING HUMAN EPIDERMAL KERATINOCYTES FROM CRYOPRESERVED AMPULES

A. Statement of Work: This method describes the procedures used to set cryopreserved ampules of human epidermal keratinocytes (HEKs) into culture, for passaging HEK cultures, and for maintaining HEK cultures.

B. Purpose: The purpose of this method is to provide the procedures needed for initiating and maintaining NHEK cultures.

C. Materials:

- 70 percent Alcohol (ethanol or isopropanol in water)
- Tissue Culture Flask (T-Flask - 25 square cm)
- Keratinocyte Growth Medium® (KGM®) - This may be purchased from Clonetics all ready prepared (only need to add supplied Bovine Pituitary Extract) or may be purchased as a KGM Bullet Kit®. The advantage of the KGM Bullet Kit® is that if left unopened the Keratinocyte Basal Medium® (KBM®) and Singlequat® growth factor component have a shelf life of 6 months. Once combined (KGM®) the medium should be used within 6 weeks.
- Hepes Buffered Saline Solution (Hepes BSS)
- Trypsin-EDTA Reagent
- Trypsin Neutralizing Solution (TNS)

D. Procedures:

1. Preparing Biological Safety Cabinet (BSC) for Sterile Use:

   a. Make sure all of the material in the BSC is sterile (for example: remove any opened containers of centrifuge tubes, etc). This is particularly true if the biological safety BSC blower fan has been turned off. Assume that any unopened item which is marked sterile is still sterile.

   b. Make sure that the BSC is operating in a laminar flow fashion (the blower fan switch on the hood should be on).

   c. With 70 percent alcohol, thoroughly wipe down the walls and floor of the BSC. If the BSC has not been used for tissue culture purposes in over a month, then remove the BSC floor, wash all sections of the hood including the both sides of the floor with a dilute Roccal solution, then with 70 percent alcohol and replace the hood floor.

   d. Allow the alcohol to evaporate prior to starting work within the BSC.

2. Personnel Clothing for Aseptic Work:

   a. Personnel who had earlier performed work in animal rooms, should at a minimum put on new scrub uniforms. Clean lab coats (not used for animal work) should also be obtained. Alternatively, white disposable labcoats may be used while performing the operations. The white labcoats, however, should not be worn outside the laboratory of operation.

   b. Exposed skin is a source of contamination, so measures to eliminate having exposed skin inside the BSC and incubators will help to minimize chances of culture contamination. For this purpose, clean laboratory coats, white sleeves, and one-pair of clean surgical gloves are worn. Safety glasses should also be worn.

3. Guidelines for Basic Aseptic Technique:

   a. Always ensure that the BSC is operational.

   b. Make sure that the BSC is not cluttered or overcrowded prior to cleaning and use. Material located near the front or back floor vents (particularly objects which are tall such as 4-L decontamination buckets) will interfere with air flow patterns and potentially redirect air flow across the floor of the hood. This may very well result in contamination during the aseptic process. Only keep the items needed for operation in the BSC and place them toward the sides and in the center (front-to-back).

   c. Clean the surface of the hood with 70 percent ethanol solution prior to use. The surface should be cleaned again after the operation is completed and before/after each operation within the same day.

   d. The hood is thoroughly cleaned as described above at least once a month (Section D.1.c.).

   e. Proper clothing is worn at all times.

   f. All operations should be performed in the center of the hood in both the front-to-back and side-to-side directions. Operations should not be performed within four inches of the front or back floor vents. Procedures should also not be performed more than 12 inches off the floor of the hood.

   g. Only sterile material is used in the operations. If there is any doubt that material (serological pipet, centrifuge tube, or T-flask) has been contaminated, then do not use the material. If a sterile serological pipet comes into contact with the outside of

a centrifuge tube, T-flask, hood floor or wall, etc., or your gloved hand accidentally touches the pipet or the inside of any sterile container, then the material is automatically considered contaminated and should not be used any further and should immediately be disposed of.

h. To the maximum extent possible, minimize the time in which containers are open. This applies to bottles of medium, sterile centrifuge tubes or T-flask containing medium or cultures, empty sterile T-flask or centrifuge tubes, etc. Only have the sterile vessels open when you are ready to use them. It is suggested that before an aseptic technique is to occur, that the lids to the vessels are loosened, the serological pipet is removed from its container (if used), then the lids to the vessels may be removed with one-hand will the sterile pipet is held with the second hand. The lids may be placed on the floor hood, only with the open end of the lid facing upward. The lid is considered contaminated if your gloved hand comes in contact with the lip or inside part of the lid.

4. Preparation of KGM® - KGM® comes as KGM® medium that must be supplemented with a vial of Bovine Pituitary Extract and also as a KGM Bullet Kit® which consists of KBM® plus a vial of growth factors (Singlequat®) when reconstituted yield KGM®. The KGM® has a shelf life of 6 weeks whereas the KGM Bullet Kit® can be stored for 6 months with the components separated.

   a. Prepare the BSC for sterile use as previously described.

   b. Remove the vial of growth factors (Singlequat®) from the -70 C freezer, place in the BSC, and allow to thaw at room temperature.

   c. When the vial has thawed, remove the KBM® from the refrigerator. Wipe the outside of the Singlequat® containers with 70 percent alcohol and aseptically using a sterile serological pipet, add the contents of the Singlequat® vial to the bottle of KBM®. Mark the bottle of KBM® as being KGM® and also initials and date the bottle and immediately return to the refrigerator. Keep the medium refrigerated as much as possible, removing the needed amounts as required. (Do not allow the bottle of medium to stand at room temperatures for prolonged periods). The KGM® should be marked with a shelf life of 6 weeks.

5. Receipt and Allocation of Reagents - The Trypsin, Hepes BSS, and Trypsin neutralizing solution will be obtained as frozen reagents. Upon receipt or shortly thereafter, the reagents should be aseptically aliquoted into sterile tubes labeled with the reagent lot number, current date, expiration date and initials. This information plus the manufacturing date and any other information supplied by the

manufacture should be kept for study record purposes. The reagents are first mixed by a series of inversions, then are aseptically aliquoted as described below and stored between -10 and -20 C.

a. Trypsin - Place 2.0 mL of solution into a 5-mL sterile polypropylene tube.

b. Hepes BSS - Place 3.0 mL of solution into a 5-mL sterile polypropylene tube.

c. Trypsin Neutralizing Solution (TNS) - Place 4 mL of solution into a 15-mL sterile conical centrifuge tube.

6. Initiating a Secondary Culture from Cryopreserved Cells - No more than one cryoampule should be thawed at a time.

a. Prepare the BSC for sterile use as previously described. Place in the hood needed material such as several 1-mL serological pipets.

b. Prewarm the needed volume of KGM® - The seeding density is 2,500 cells per square centimeter of growth area. As there are approximately 500,000 cells per cryoampule, the total volume of KGM® required will be 40 mL and will be cultured in 6-25 square cm T-flask. Label six 25-square cm T-flasks as being secondary (2') keratinocyte (HEKs) cultures, the keratinocyte lot or strain number, 2,500 cells per square cm, date 2' culture initiated, and initials. This information along with the lot number of the media used should be recorded as part of the study notes. Mark a seventh flask as KGM. Remove the KGM from the refrigerator as previously described and place into the BSC. Aseptically, add 5 mL of KGM® to each flask. Tighten the flask caps, then transfer to a 37 C, 5 percent carbon dioxide incubator, where the caps are loosened by one-full revolution while the flasks are held in the incubator. The flask with media should be allowed to equilibrate for a minimum of 30 min before use. Immediately return the bottle of KGM® to the refrigerator.

c. Approximately 50 mL of 70 percent ethanol in water is placed in a 100-mL beaker, covered with parafilm, and then brought to 37 C (place in 37 C incubator for 1 hr). The ethanol must be brought to 37 C prior to removing the cryopreserved HEKs from the liquid nitrogen storage dewar.

d. The cryopreserved HEK ampule is removed from the liquid nitrogen storage dewar and placed into the BSC.

e. Remove the label from the cryoampule, then submerge the container in warm (37 degree C) ethanol solution to thaw. The contents of the cryoampule should be constantly mixed when in the ethanol

solution (accomplished by moving ampule back and forth in the beaker). When most of the material in the ampule has thawed, the ampule is removed and the remainder of the frozen material is thawed by a series of cryoampule inversions.

f. As soon as the material has thawed, remove the T-flask from the incubator by tightening the caps while in the incubator and placing them in the BSC. Immediately, loosen the cap of the T-flask marked as KGM® and the cap of the cryoampule. With a 1-mL sterile serological pipet remove the contents and add to the T-flask containing the 5 mL of KGM®. The contents of the T-flask are mixed and 1 mL of the cell suspension is added to each of the six other T-flasks.

g. The T-flasks are tightly capped, and the contents of the flasks are gently rolled back and forth across the bottom of the container to mix the suspension so that even dispersement of the cells over the growth surface occurs. Take extreme care at all times not to allow medium to come into contact with the neck or opening of the T-flask as this will increase the chance of culture contamination.

h. With the cap tightly in place, transport the T-flask to the incubator, place the T-flask with the growth surface down, and slightly opened the caps (about one full turn). If the caps are left secured, then no gas exchange will occur and the culture will not be viable. If the caps are loosened to far (to the point where they are only hanging on the neck), then there is a good chance of culture contamination.

i. After 24 hr, replace the spent medium with fresh, prewarmed medium. T-flasks are removed by first tightening the caps while still in the incubator, rotating the T-flask to an upright position so the medium is at the bottom, then transferring the T-flask to the BSC (place the growth surface down in the BSC). Replace the entire volume of spent medium by holding the T-flasks upright, removing the cap, then aspirating the spent medium from a upper-bottom corner of the flask (corner not in contact with the growth surface). Using a clean, sterile serological pipet, add the appropriate amount of fresh media to the flask (5-9 mL as directed for 25 square cm T-flask), recap the T-flask, and return to the incubator using the technique described above. Every other day thereafter, this procedure of replacing the spent medium should be performed.

7. Trypsinizing Cells Contained in a 25 Square Cm T-Flask - This should be performed for cultures between 70 to 85 percent confluent as assessed microscopically. Cultures over 85 percent confluent will start to differentiate which may results in poor growth.

a. Prepare the BSC for sterile use as previously described.

b. Remove the Hepes BSS, Trypsin-EDTA, and TNS from the freezer and place them in the BSC to thaw. These reagents must be allowed to equilibrate to room temperature before they are used (This will take an approximate 1.5 hrs at room temperature).

c. Aseptically add the needed quantity of KGM® into a tissue culture flask, place in a 37 degree, 5 percent carbon dioxide incubator, and loosen the cap to allow gas exchange within the T-flask. The medium should be allowed to warm to 37 degrees and equilibrate with the incubator's gaseous atmosphere for at least 30 min.

d. Place all necessary equipment (serological pipets - 1 and 5 mL, Coulter Vial, stop watch) in the BSC.

e. Once all the reagents have equilibrated, remove the T-flask containing the cells from the incubator by first tightening the T-flask lid while still in the incubator, then moving the T-flask to the BSC being careful not to get tissue culture medium near the cap or neck of the T-flask. Place the T-flask (growth surface down) in the BSC. At all points be careful to not allow the growth surface to become dry as this will result in loss of cell viability.

f. Stand the T-flask on end, remove the cap, and aspirate as much of the spent tissue culture medium from the bottom/top section of the flask as possible.

g. With a clean and sterile serological pipet, add 2 mL of the Trypsin-EDTA reagent to the T-flask, place the cap on tightly, orient the flask so that the fluid is in contact with the cells, and immediately start the stop watch.

    1) Remove the flask from the BSC and place on the inverted microscope. Observe the cells using the 10- or 20-X objective.

    2) Allow the trypsinization process to continue until 50 percent or more of the cells have detached or become rounded (3 to 4 min exposure to Trypsin-EDTA). At this point, rap the flask (growth surface side first) against the palm of your hand, then turn the flask over and rap the top of the flask against the palm of your hand.

    3) Immediately, examine the flask under the microscope. If at least 90 percent of the cells have not released, allow the trypsinization process to continue (DO NOT allow the trypsinization process to proceed for longer than 7 min).

Check the progress of detachment every 30 to 60 seconds. If the cells will not detach, remove the Trypsin-EDTA, wash the cells with HEPES BSS, add some KGM to the flask, obtain Trypsin-EDTA solution from a new container, and try again.

4) If the majority (over 80 percent) of cells have detached, immediately transfer the flask to the BSC. With a sterile 5-mL pipet, immediately wash the top and bottom surface of the flask with 3-mL of HEPES BSS. Aspirate all contents of the flask and immediately add to the centrifuge tube containing 4 mL of TNS.

h. Centrifuge the sample at 220 x g (1,000 rpm) for a 5-min period at 25 degrees C. Gently aspirate as much of the supernatant as is possible without disrupting the cell pellet, then resuspend the pellet with 2 to 5 mL of prewarmed KGM® (always include the volume in the study notes). With a sterile 1-mL serological pipet transfer 100 µL of cell suspension to a small tube and perform a cell count using a Hemacytometer or Coulter Counter as follows.

1) Hemacytometer - This procedure does not require aseptic technique). Make sure that the hemacytometer (coverslip included) is clean. Place the cover on the hemacytometer and fill both hemacytometer chambers with cell suspension (requires about 30 µL per side). Count the number of cells contained in three of nine the 1 by 1 mm areas (see Attachment A, schematic of Hemacytometer). Cells which contact the top and right side lines are not included in the count, while those contacting the bottom and left side are included. Record the number counted for the three 1 by 1 mm area, then examine the second chamber repeating this process. Calculate the average of the six values, then multiply by 10,000 to obtain the number of cells per mL of suspension. Clean the hemacytometer shortly after use, first with soap and water, then with distilled water. Record this value and use it in the calculations shown below.

2) Coulter Counter - Prior to performing a cell count, you should verify that the Coulter background is below 100. A cell count is performed by placing 10 mL of sterile counting solution into a counting vial, adding 50 µL of cell suspension, mixing, and then counting three times. The cell count is the average of the three readings divided by 2.5, and the units for this cell count are 10^6 cells per mL of cell suspension. Make the appropriate addition of cell suspension and diluent (prewarmed tissue culture medium) in a sterile container. Mix well and seed to the microtest plate as experimentally required. If the seeding process is more than two plates, the cell suspension should be mixed again by

gentle repeated refluxing before seeding the next set of microtest plates. The formula for calculating the volume of cell suspension and diluent to obtain the target cell density is as shown in the following section.

i. Calculations

1. Calculate the Dilution Factor:

$$\text{Factor} = \frac{\text{Cell Count}}{\text{Target Cell Density}}$$

2. Calculate the Total Number of Cells:

$$\text{No. Cells } (\times 10^6) = \text{Cell Count} \times \text{Vol}_{\text{cell susp.}}$$

where,

$\text{Vol}_{\text{cell susp.}}$ = volume of resuspended cell pellet to be diluted.

3. Calculate the Total Volume Required:

$$\text{Vol}_{\text{total}}\ (\text{mL}) = \frac{\text{No. Cells } (\times 10^6)}{\text{Target Cell Density } (\times 10^6/\text{mL})}$$

4. $\text{Vol}_{\text{diluent}} = \text{Vol}_{\text{total}} - \text{Vol}_{\text{cell susp.}}$

j. Generally the seeding density is 2,000 to 3,000 cells per well. This translates to 6,250 to 9,375 cells per square centimeter of growth surface for the microplate systems. The target cell density required for seeding can be obtained from the following table.

Culture System	Number of Cells	Culture Volume (mL)	Number of Cells Per mL
	6,250 cells/cm^2		
96-well Plate	2,000/well	0.2	10,000
24-well Plate	9,140/well	1.0	9,140
	7,810 cells/cm^2		
96-well Plate	2,500/well	0.2	12,500
24-well Plate	11,403/well	1.0	11,403
	9,375 cells/cm^2		
96-well Plate	3,000/well	0.2	15,000
24-well Plate	13,688/well	1.0	13,688

1. Personnel Training - Personnel training will proceed following this method. Individuals who are considered to have been successfully trained will have demonstrated successful initial seeding of keratinocyte cultures, maintenance of cultures, and successful subculturing of cells. This training will be performed under the supervision of James Blank or Rebekah Starner.

Originated by: James A. Blank
James A. Blank, Ph.D.
Principal Research Scientist

Feb 15, 1993
Date

Reviewed by: Rebekah A. Starner
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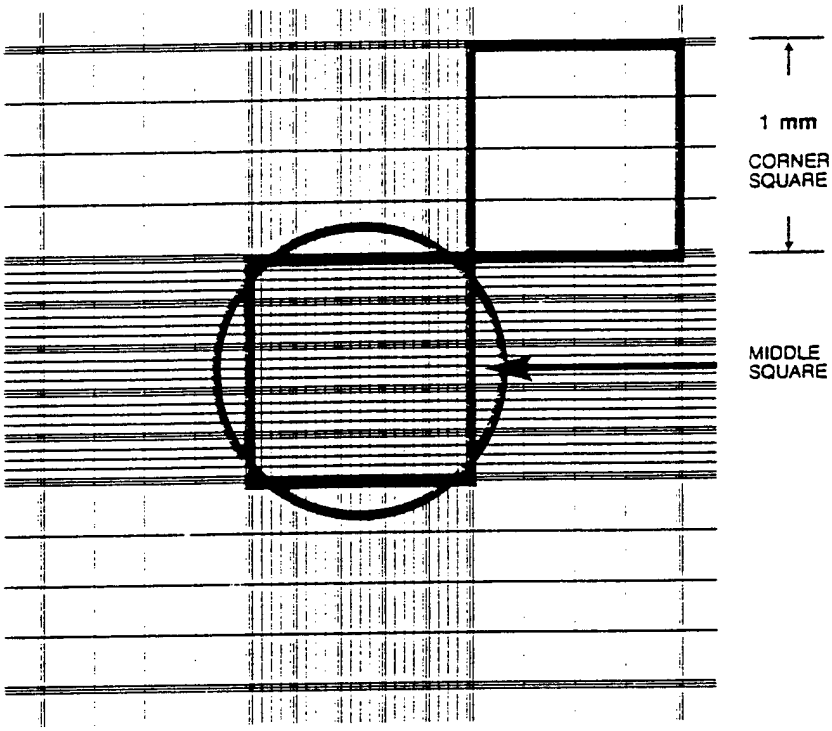
Feb 15, 1993
Date

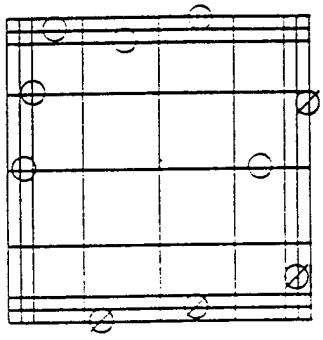
ATTACHMENT A

STANDARD HEMOCYTOMETER CHAMBER



The circle indicates the approximate area covered at 100× microscope magnification (10× ocular and 10× objective). Include cells on top and left touching middle line ( ). Do not count cells touching middle line at bottom and right ( ). Count 4 corner squares and middle square in both chambers (one chamber represented here).

DIAGRAM III
CORNER SQUARE (ENLARGEMENT)



Count cells on top and left touching middle line ( ). Do not count cells touching middle line at bottom and right ( ).

ATTACHMENT C

Battelle SOP MREF V-011

Manual Number: MREF. II

Battelle SOP Number: MREF V-011-00

Key Words: sulfur mustard,
in vitro, cell culture

STANDARD OPERATING PROCEDURE (SOP) FOR EXPOSURE OF CELL CULTURES TO SULFUR MUSTARD TO ASSESS THE IMPACT UPON CELLULAR AND BIOCHEMICAL ENDPOINTS

Originated by: James A. Blank
James A. Blank, Ph.D. Date 07 Oct 1991

Approved by: _____
Business Unit Manager Date 10/9/91

Approved by: David Stilk
CIH, Safety and Surety Officer Date 10/11/91

Reviewed and Registered by QAU: M Patterson Effective Date 10-14-91

Distribution List:

Quality Assurance Unit
SOP Manual(s)

Battelle
Health and Environment Group
505 King Avenue
Columbus, Ohio 43201-2693

I. Scope

This standard operating procedure (SOP) describes the procedure for exposing cell cultures to sulfur mustard (HD), and pretreatment and therapeutic (P&T) compounds.

II. Purpose

HD has been shown to decrease normal human epidermal keratinocyte and human leukocyte viability and nicotinamide adenine dinucleotide 2 (NAD^+, oxidated form) following in vitro exposure (see Section III). Thus, cellular viability as well as cellular NAD^+ levels and potentially other biochemical parameters should provide a useful endpoint for assessing in vitro exposure to HD and for evaluating the effectiveness of candidate P&T compounds to counteract the cytotoxic effect of HD.

III. References

Battelle SOP MREF I-002, "Standard Operating Procedure for the Storage, Dilution, and Transfer of GA, GB, GD, TGD, VX, HD, HD/L, and L When CSM Concentration/Quantity is Greater Than Exempt Levels".

Battelle SOP MREF I-003, "Standard Operating Procedure for Receipt, Transfer, Storage, and Use of Exempt Chemical Surety Materiel (XCSM) (e.g., XGA, XGB, XGD, XTGD, XVX, XHD, XHL, and XL)".

Battelle SOP MREF V-009, "Standard Operating Procedure for the Collection and Use of Human Blood".

Meier, H.L., C.L. Gross, and B. Papirmeister, 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard) Decreases NAD^+ Levels in Human Leukocytes, Toxicol. Lett. 39:109-122, (1987).

Smith, W.J., C.L. Gross, P. Chan, and H.L. Meier, The Use of Human Epidermal Keratinocytes in Culture as a Model for Studying the Biochemical Mechanisms of Sulfur Mustard Toxicity, Cell Biol. Toxicol. 6:285-291, (1990).

IV. Definitions

HD - Sulfur Mustard (CAS 505-60-2 or 39472-40-7 or 68157-62-0): 2,2'-Dichloroethyl Sulfide.

XHD - Exempt level of HD also referred to as research, development, test, and evaluation (RDTE) dilute solutions of HD. XHD are those concentrations of HD that do not exceed the concentration or volume limits described in Battelle SOP MREF I-003.

V. Procedures

A. Preparation of Cells

1. The tissue culture supernate is slowly aspirated from the NHEK cultures in the multiwell plates with care taken not to disturb the monolayer cultures and a small volume of tissue culture media is added to prevent drying of the cultures.

2. Human peripheral leukocytes are isolated from blood samples using a density gradient separation method. Human blood specimens are handled following Battelle SOP MREF V-009. If other non-adherent cell types are used, then the protocol specified number of cells are added to each well.

B. Addition of P&T Compound

Varying volumes of P&T compound or its vehicle (as the control) in tissue culture medium are added to the appropriate wells so that the protocol stated P&T concentration are achieved. Prior to P&T compound addition, 1640 is added to each well so that upon addition of P&T compound the volume in each well is the same. P&T compound is added at the protocol defined time. A positive control compound such as niacinamide (NM) may also be used for assay control purposes as defined by the protocol.

C. Dilution of HD to XHD

HD is diluted into a 20-mL scintillation vial to a final HD concentration of 0.48 mg/mL (3 mM) following Battelle SOP MREF I-002. The diluent is ice cold saline, tissue culture medium, or as specified by the protocol.

1. A shallow container is filled to approximately 1 inch with a methanol dry ice mixture. The scintillation vials, which have been clearly labeled to indicate the contents, concentration, date of preparation, and the name of the individual preparing the dilution, are contained in a rack situated in the methanol dry ice mixture. Five microliters of HD is added to each vial.

2. Ice cold diluent (13.3 mL) is added to each vial. When the solution freezes, the vials are packaged for storage as described in Battelle SOP MREF I-003 and transferred to a -70 degree C freezer for storage.

3. For each XHD vial, a vehicle control vial is prepared. This consists of the diluent lot used for XHD preparation that has been frozen in a methanol dry ice bath. This vehicle control should be prepared on the same day as the XHD.

4. Alternatively, HD may be diluted in a 20-mL scintillation vial to a final HD concentration of 0.32 mg/mL into ice cold tissue culture media or as specified by the protocol following Battelle SOP MREF I-002. The vial is clearly labeled to indicate the contents, concentration, date of preparation, and the name of the individual preparing the dilution.

D. **Dilution of XHD**

The XHD is further diluted to the desired or target concentration following the procedures outlined in Battelle SOP MREF I-003. The dilution factor, volume of 3 mM XHD, and volume of ice cold diluent which are used, are calculated as follows:

1. The dilution factor is calculated as follows:

$$\text{Dilution factor} = \frac{3\ \text{mM}}{\text{Target Concentration (mM)}}$$

2. The volume of stock (3 mM) XHD solution to be diluted is calculated as follows:

$$\text{Volume 3 mM XHD} = \frac{\text{Volume}_{\text{total}}}{\text{Dilution Factor}}$$

3. The volume of diluent is calculated as follows:

$$[\text{Volume}_{\text{total}} - \text{Volume of 3 mM XHD}] = \text{Diluent Volume}$$

E. **Addition of XHD to Multiwell Test Plates**

The lid of the multiwell test plate is removed and XHD solution is added to the designated wells using procedures outlined in Battelle SOP MREF I-003. Upon completion of all XHD additions, the plate is allowed to set in the biological safety cabinet for 1 hr prior to replacing the multiwell plate lid.

F. Incubation of Multiwell Test Plates

1. The plates are transferred to a carbon dioxide incubator for the protocol specified length of time, and at the protocol specified temperature.

2. After incubation, the multiwell test plate is removed from the incubator and transferred to the biological safety cabinet.

G. Endpoint

The samples are then analyzed for the protocol specified cellular and/or biochemical parameter.

BATTELLE PROPRIETARY

METHOD FOR THE DETERMINATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) USING THE MULTISKAN MCC 340 MK II ELISA PLATE READER

A. Statement of Work: This method describes the procedures used to measure nicotinamide adenine dinucleotide (NAD^+) levels in cellular samples. This procedure described in this method for NAD^+ analysis is based upon that of Jacobson and Jacobson (Section H.3.) adapted for use in a 96 well microtiter plate system.

B. Abbreviations and Reagents:

1. ADH - Alcohol Dehydrogenase (Sigma #A3263; 280 U/mg solid)
2. BSA - Bovine Serum Albumin
3. EDTA - Ethylenediamine tetraacetic acid
4. EtOH - Absolute ethanol
5. HCl - Hydrochloric Acid
6. $HClO_4$ - Perchloric Acid
7. KPO_4 - Potassium Phosphate
8. KOH - Potassium Hydroxide
9. MTT - MTT Tetrazolium
10. NAD^+ - Nicotinamide Adenine Dinucleotide (oxidized)
11. PES - Phenazine ethosulfate

C. Precautions:

1. $HClO_4$ - $HClO_4$ is a potent oxidizing and caustic agent. Its use in the presence of organic solvents must be avoided. Dilutions of stock $HClO_4$ (60 - 70 percent $HClO_4$ solution) must be made in the biological safety cabinet as should all work with diluted $HClO_4$ solution.

2. MTT and PES - These are labeled as being suspect carcinogens. They should be treated as such and weighed on a scale located in a vented cabinet. These are also light sensitive chemicals and should be weighed under reduced lighting. The container in which they are solubilized should be enclosed in foil.

D. Preparation of Reagents:

1. $HClO_4$ Dilution - All dilutions and additions of $HClO_4$ are to be performed in a biological safety cabinet that does not contain any organic solvents. The procedure requires two individuals. $HClO_4$ can be obtained as an approximate 60 to 70 percent solution in water. The stock bottle is kept in a secondary container stored in a cabinet that is void of any organic acids or solvents. The molarity of the stock $HClO_4$ solution is calculated as follows:

a. $$\frac{(1.768 \text{ gm/mL}) \times (1{,}000 \text{ mL/L})}{100.42 \text{ gm/mol}} = X$$

b. X multiplied by the percent of the $HClO_4$ solution and divided by 100 gives the molarity of the stock $HClO_4$ solution.

Prepare a 3 M $HClO_4$ solution in the biological safety cabinet over plastic backed paper using distilled water as the diluent. The solution is not used 60 days past the date of preparation.

c. One individual will remove the $HClO_4$ from the secondary container, open the cap of the $HClO_4$, and recap the bottle as soon as the aliquot is removed. The second individual removes and transfers the volume of stock $HClO_4$ to the Erlenmeyer flask. The $HClO_4$ is dispensed below the level of the distilled water. Just after dispensing, the serological pipette is filled with the diluted acid solution and this material is dispensed back into the Erlenmeyer flask. The Erlenmeyer is stoppered and clearly labeled with the following information: contents, molarity, date of preparation, and individual making the solution. The 3 M $HClO_4$ solution is kept refrigerated and will not be used 60 days past the date of preparation. Prepare 0.5 m $HClO_4$ by adding 30 mL of 3 M $HClO_4$ to 150 mL of distilled deionized water. The first individual replaces the $HClO_4$ in its secondary container and returns the secondary container to the acid cabinet.

2. Preparation of KOH/KPO_4 Buffer:

a. Dissolve 28.74 gm of dibasic KPO_4 (K_2HPO_4) in 400 mL of distilled water contained in a 500-mL beaker and adjust the pH to 7.8 with 3 N HCl.

b. Dissolve 28.05 gm of KOH in the phosphate buffered solution prepared above in Section 2.a., transfer the solution to a 500-mL volumetric flask, and fill to the 500 mL volume mark with distilled water. The solution contains 0.33 M potassium phosphate and 1 M potassium hydroxide. The container will be labeled identifying the contents of the container, date of preparation, and the initials of the individual making the solution.

c. The KOH/KPO_4 buffer is used to neutralize the $HClO_4$ solution. As slight variation in the $HClO_4$ and KOH/KPO_4 solution may exist when the solutions are prepared, it is necessary to titrate the $HClO_4$ solution with KOH/KPO_4 when either of the solutions is prepared. Twelve mL of 0.5 m $HClO_4$ os added to a 20 mL scintillation vial. Five mL of KOH/KPO_4 buffer is added to the $HClO_4$, the solution mixed and the solution pH determined. Fifty μL aliquots of KOH/KPO_4 buffer is added to the scintillation vial until the resulting solution pH is between 6.8 and 7.0. The cumulative or total volume (mL) of KOH/KPO_4 buffer required to achieve this final pH (6.8 to 7.0) is computed. Divide this value by 100 yields the μL volume of KOH/KPO_4 required to neutralize 120 μL of 0.5M $HClO_4$ solution. This volume should be 60 μL $\pm$ 5 μL. This volume, along with the date is marked on the $HClO_4$ and KOH/KPO_4

containers. If outside this range, either the $HClO_4$ or the KOH/KPO_4 solutions should be prepared.

d. The solution is kept refrigerated and will not be used 60 days past the date of preparation.

3. Preparation of Assay Buffers:

a. Buffer I:

Reagent	Concentration	GM/100 mL
Bicine	200 mM	3.264
EDTA	8.32 mM	0.310
BSA	1.66 mg/mL	0.166

Adjust to pH 7.8 with 1N KOH. Mark date of preparation, initial of individual preparing solution, and the expiration date which is 2 months when stored refrigerated.

b. Buffer II:

Reagent	Concentration	GM/20 mL
MTT	0.84 mM	0.0068
PES	6.64 mM	0.0444
EtOH	500.00 mM	

(1) Prepare just prior to use. Dissolve the MTT and PES in 18.8 mL of Buffer I. Then add 1.2 mL of EtOH and mix. This is performed under reduced lighting.

(2) Wrap the container with aluminum foil immediately after preparation to protect from lighting. Discard solution after use.

4. Preparation of ADH - Prepare using distilled deionized water on the day of use and store refrigerated. Add 0.004 gm of ADH to 7.5 mL of diluent to yield a 150 U/mL solution. Discard solution after use.

5. Preparation of NAD^+ Standards:

a. 1.5×10^{-3} M Stock: Add 0.0054 gm of NAD^+ to 5 mL of 0.5 M $HClO_4$ and mix. Use 0.5 M $HClO_4$ as the diluent in subsequent dilutions.

b. 1.5×10^{-5} M (Working Stock): Add 0.05 mL of a. to 5 mL of diluent and mix.

c. 1.13×10^{-6} M: 0.75 mL of b. + 9.25 mL diluent. (750 nM)

d. 7.5×10^{-7} M: 4 mL of c. + 2 mL diluent. (500 nM)

e. 3.75×10^{-7} M: 3 mL of d. + 3 mL diluent. (250 nM)

f. 1.88×10^{-7} M: 3 mL of e. + 3 mL diluent. (125 nM)

g. 9.4×10^{-8} M: 3 mL of f. + 3 mL diluent. (62.5 nM)

h. 4.7×10^{-8} M: 3 mL of g. + 3 mL diluent. (31.25 nM)

i. 2.3×10^{-8} M: 3 mL of h. + 3 mL diluent. (15.625 nM)

j. Background: 0.5 M perchloric acid used for diluent.

Prepare the dilutions of the standards and place 0.5-mL aliquots into a series of Eppendorf centrifuge tubes. The tubes should be labeled indicating the standard letter, date of preparation, and individual preparing the dilution. The standards can be used for up to 1 month following preparation when stored frozen.

6. Preparation of Sodium Iodoacetate:

A 120 mM solution of sodium iodoacetate is prepared by adding 1.248 gm to 50 mL of distilled water. The solution container is marked to indicate contents, a 30-day expiration date, and the name of the individual preparing the solution.

E. Instrument and Computer Power Up : Titertek Multiskan MCC/340 ELISA Plate Reader is used in accordance with Battelle SOP MREF V-002.

1. For electronic data capture, turn on the computer and go into the communications mode of the SYMPHONY Spreadsheet for NAD^+ analysis prior to powering on the ELISA reader.

2. Press the "Send ABS" key on the ELISA plate reader to electronically transfer a blank into the Symphony spreadsheet for the proper spreadsheet setup.

3. Return to the Sheet Mode of the spreadsheet, and press the F7 key, then type "ERASE", then return the spreadsheet to the communications mode.

4. On the ELISA Reader, Press Measurement Mode - press arrow key until "single wavelength absorbance" appears on screen and press enter.

5. Using the numerical keys, press 540 and enter.

6. Press arrow key until "no background" appears on screen and press enter.

F. Extraction of NAD^+:

1. The microtiter plates are removed from the tray and the microtiter plate lids removed. Twenty μL of 3 M $HClO_4$ is added to each well. The lids are replaced on the microtiter plates and the samples are refrigerated for 30 min. The cap is replaced on the $HClO_4$ and returned to the refrigerator. (At this point, parafilm may be placed over the plate and the plate transferred to the -70 degree freezer until further analysis.)

2. The plates and the container of KOH/KPO_4 buffer are transferred from the refrigerator to the hood in Room 47A and the volume of KOH/KPO_4 buffer determined in the titration step in Section D.2.c is added using a multichannel pipette. The lids are replaced on the plates and the plates returned to the refrigerator for a minimum of 15 min.

3. The covered plates are placed into microplate carriers in the hood in Room 47A, balanced, then centrifuged at 1,200 x g (2,500 rpm) for 10 min.

G. Analysis of NAD^+:

1. Thaw a set of NAD^+ standards, add 250 μL of the KOH/KPO_4 solution, vortex, then incubate in the refrigerator for 15 min. Centrifuge the standards for 10 min at a minimum of 1,000 x g.

2. Add 75 μL of background (0 nM NAD^+ standard) and NAD standards (described in D.5.c through D.5.j) to each of four wells. Assay the background and standards on a plate separate from the samples.

3. To assay the samples, transfer 40 to 75 μL of sample to a new 96 well microtiter plate. If less than 75 μL is used then make up the difference with assay buffer.

4. Add 75 μL of Buffer II to each well under dimmed lights.

5. Add 20 μL of ADH (150 U/mL) under dimmed lights to initiate the reaction.

6. Place plate in 37 degree C in incubator for 15 min. Remove plate from incubator, then immediately add 20 μL of sodium iodoacetate (120 mM) solution. (Be sure to wipe the bottom of the plate thoroughly with lens paper to remove any condensation.)

7. Immediately measure the absorbance of the wells at 540 nm.

8. Insert the microtiter plate onto the reader platform and press start. The Titertek will print out the absorbance values. This printout will be appropriately identified, initialed, and dated. The printout must be copied and initiated for the study records as the printout will fade with time.

9. Press the "SEND ABS" key to electronically transfer the data into the Lotus Symphony spreadsheet program.

10. Perform steps (8) to (9) first with the standards to ensure that no problems exist with the reagents. Then proceed to assay the samples.

H. Calculation of NAD^+:

1. Regression analysis is performed on the standards to obtain slope (m) and y-intercept (b) values. Nanomolar concentrations of NAD standards are the independent (x) variable and background subtracted absorbance values at 540 nm are the dependent (y) variable.

2. Background subtracted absorbance values at 540 nm for samples are converted to picomol NAD per sample using the following series of calculations:

(a) Line equation $x = (y - b)/m$

where: m = slope
b = y-intercept
y = background subtracted absorbance at 540 nm
x = NAD (nM)

(b) $\frac{\text{nmol NAD}}{\text{L}} * \frac{1\text{L}}{10^6\ \mu\text{L}} \times \frac{10^3\ \text{pmol}}{\text{nmol}} \times \mu\text{L Sample Volume}$

* dilution factor(s)

I. References:

1. Mol, M.A.E., A.B.C. Van De Ruit, and A.W. Kluivers, NAD^+ Levels and Glucose Uptake of Cultured Human Epidermal Cells Exposed to Sulfur Mustard, Tox. App. Pharmacol. 98:159-165 (1989).

2. Meier, H. L., C.L. Gross, and B. Papirmeister, 2,2'-Dichlorodiethyl Sulfide (Sulfur Mustard) Decreases NAD^+ Levels in Human Leukocytes, Tox. Lett. 39:109-122 (1987).

3. Jacobson, E.L. and M.K. Jacobson, Pyridine Nucleotide Levels as a Function of Growth in Normal and Transformed 3T3 Cells, Arch. Biochem. Biophys. 175:627-634 (1976).

Originated by: James A. Blank — 12 Jan 1994
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Date

Reviewed by: Rebekah A Starner — 11 Jan 1994
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Researcher
Date

Manual Number: MREF.11

Battelle SOP Number: MREF V-012-00

Key Words: PADPRP, ^{3}H-NAD^{+}

STANDARD OPERATING PROCEDURE (SOP) FOR ASSESSING POLY(ADP-RIBOSE) ACTIVITY FROM SULFUR MUSTARD EXPOSED CELLS AND TISSUES USING TRITIATED-NICOTINAMIDE ADENINE DINUCLEOTIDE

Originated by: James A. Blank, Ph.D. Date 23 July 1993

Approved by: David W. Hobson, Ph.D., D.A.B.T.
Principal Investigator and Manager
Medical Research and Evaluation Facility Date 7/26/93

Approved by: CIH, Safety and Surety Officer Date 26 July 93

Reviewed and Registered by QAU: Effective Date 7-29-93

Distribution List:

Quality Assurance Unit
SOP Manual(s)

Battelle
505 King Avenue
Columbus, Ohio 43201-2693

I. Scope

This SOP describes the receipt, use, and disposal of radiolabeled material used for determining the activity of poly(ADP-ribose) polymerase (PADPRP) activity in biological samples.

II. Purpose

Sulfur mustard (HD) binds to cellular genetic material and induces damages through irreversible binding. PADPRP activity is increased in response to this damage in an attempt to inhibit repair. PADPRP activity catalyzes the transfer of the ADP-ribose portion of nicotinamide adenine dinucleotide (NAD^+) molecule to proteins which regulate repair of the damaged genetic material. As the radiolabeled residues in the ADP-ribose portion of NAD^+, PADPRP activity can be measured by incubating cellular homogenates or nuclear preparations with tritiated NAD^+ (^3H-NAD^+) precipitating the modified proteins, and determining the amount of radiolabel incorporated.

III. References

Battelle SOP Medical Research and Evaluation Facility (MREF) I-003, "Standard Operating Procedure for the Receipt, Transfer, Storage, and Use of Exempt Chemical Surety Materiel (XCSM) (e.g., XGA, XGB, XGD, XTGD, XVX, XHD, XHL, and XL)".

Battelle Columbus Operations Radiation Protection Plan, January 1993.

Battelle SOP H/SP III-001, "Standard Operating Procedure for the Procurement and Receipt of Radioactive Materials".

Battelle SOP H/SP III-002, "Standard Operating Procedure for Medical Surveillance and Personnel Training Requirements for Personnel Working with Radionuclides".

Battelle SOP H/SP III-003, "Standard Operating Procedure for the Storage and Use of Radioactive Materials".

Battelle SOP H/SP III-004, "Standard Operating Procedure for Radiation Monitoring".

Battelle SOP H/SP III-005, "Standard Operating Procedure for the Disposal of Radioactive Waste and Contaminated Equipment".

Material Safety Data Sheets (MSDS) are available in the administrative area of the MREF or through Battelle's Safety Office, 505 King Avenue, Columbus, Ohio.

IV. Definitions

A. **Radioactive Material Log** - A record log, kept separate from study records and maintained by the Principal Investigator, to account for radioactive material. This log which is maintained following guidance provided in the Radiation Protection Plan is as shown in Attachment 1.

B. **Laboratory Monitor Log** - A record log of laboratory monitoring for contamination and results obtained for monitoring process. The monitoring process is smear tests and records of the location of smears and results should be maintained using the form shown in Attachment 2.

C. **Radiation Material Application (RMA)** - Standard blank RMA forms are available through the Radiation Safety Office. RMAs are completed by the Principal Investigator and submitted to the Radiation Safety Office. The RMA must by approved prior to ordering any radiolabeled material.

V. Procedures

A. Materials to be Used

^{3}H-NAD^{+}

HD

B. Hazards Involved

1. HD is a bifunctional alkylating agent that can cause vesicating lesions to the skin, eyes, and respiratory tract. HD is also mutagenic and carcinogenic. The hazards involved in working with HD are as described in Battelle SOP MREF I-003. All procedures involving HD-exposed samples are performed in accordance with this SOP.

2. NAD^{+} does not present a hazard. The tritium tag on the NAD^{+} is a weak beta particle emitter, however, the energy emitted is so weak that penetration of the skin is negligible. Internalization of ^{3}H-NAD^{+} is of concern, and the primary routes of entry are considered to be oral ingestion, inhalation, autoinoculation, dermal and ocular exposure.

3. Trichloroacetic Acid (TCA) - This is a corrosive material that can cause severe burns. Avoid contact with eyes, skin, and clothing. Hands should be thoroughly washed following

operations involving TCA. In case of contact, immediately flush with copious amounts of water for at least 15 min.

C. Personnel Requirements and Monitoring

1. Personnel requirements for working with radioactive material are as outlined in Battelle SOP H/SP III-002. These requirements include yearly physicals and completion of a Radiation Safety Course provided by the Environment, Safety, and Health Department. Pregnancies occurring during any time of the operation must be immediately reported to the supervisor.

2. Personnel with open skin wounds should not work with unsealed radioactive material without an adequate waterproof covering on the wound and the approval of the Medical Officer.

3. Personnel monitoring is performed following Battelle SOP H/SP III-002. For ^{3}H-NAD^{+}, the use of film badge and Ludlum monitors is not appropriate for monitoring millicurie tritium exposure since the very low energy particles emitted are not a significant external hazard and cannot penetrate the dosimeter casing. Monitoring will be performed using urine bioassays. Prior to initiating any work, a baseline urine sample for individuals involved in operations using radiolabeled material is taken for bioassay. If, at any point during assay operation, a suspected exposure to ^{3}H-NAD^{+} has occurred, a urine specimen is taken three to four hours following the suspected exposure and submitted for bioassay. Upon completion of the task, a urine specimen from individuals involved in the radiolabeled procedures is submitted for bioassay.

4. If protective clothing is suspected of being contaminated, it should be surveyed by smear test. Any surgical gloves used in the process are automatically considered contaminated and should be disposed of as solid radioactive waste. Both sets of gloves should also be automatically changed prior to removing hands from a hood and every 20 min during operation.

D. Equipment

The equipment used within this SOP includes that listed in Battelle SOP MREF I-003 for any work involving HD. Additional equipment includes the following: spill trays, vacuum pump, filtration manifold, filter paper, pipets and tips, freezer (Revco) (locked), latex gloves, labels, first aid kit, plastic-backed, absorbent paper, brown paper, 4-L beakers, squirt bottles, wiping tissues, tissue paper, laboratory coat, safety shoes, protective eyewear, syringes, needles, forceps, radioactive waste bags

(4mm polyethylene), sharps box, agent vial block, inline charcoal filter, 7 mL scintillation vials, tygon tubing, syringes, needles, disposable sleeves, 20-mL scintillation vials, scintillation fluid, and a scintillation counter. Personal protective equipment for use with radioactive material is the same as for exempt levels of HD with the exception that disposable lab coats are used in place of cloth lab coats and shoe covers are used.

E. Facility Preparation

1. The RMA is conspicuously posted in the areas which are designated in the RMA where work is to be performed and signs indicating the Radioactive Hazard as described in Battelle SOP H/SP III-001 are placed on the doors of laboratories where the radiolabeled material is to be stored or used. The Radiation Safety Office will initially post signs where the material is to be stored and used.

2. Hoods are prepared for radioactive material use by a minimum of covering the hood floor first with a layer of plastic-backed paper then with a layer of brown paper. Radiolabeled tape is used to delineate the area in which the procedure is to occur. A spill tray is placed in the demarcated work area. All material and equipment necessary for operation including disposal bags (4-mil thickness polyethylene type) for solid radioactive waste are placed in the hood prior to operations. If HD is used in the same operation, then hood preparation must also follow procedures outlined in Battelle SOP MREF I-003.

F. Receipt of ^{3}H-NAD^{+}

1. A RMA must be approved by the Radiation Safety Office prior to ordering any radiolabeled material. All radioactive material should be addressed to the Radiation Safety Officer as well as contain the RMA number on the label. Upon receipt, Radiation Safety Services performs a receipt survey of the packages and adds the material received to the inventory form.

2. The Radiation Safety Officer is notified, using the form shown in Attachment 3, that the material must be transferred to the MREF, West Jefferson site.

3. Upon receipt, the container is transported to an appropriately designated room, placed inside a designated hood prepared for radioactive material use, and the packaging and secondary containers are inspected by an individual wearing appropriate personal protective equipment for any signs of damage following the procedures outlined in Battelle SOP H/SP III-001. If there

are any signs of damage, the Radiation Safety Office is immediately contacted.

4. The secondary container is removed from the package and smears are taken to assess whether the secondary container has external radioactive contamination. Smears are taken according to Battelle SOP H/SP III-004 and the results of the smear test are recorded in the radioactive material logbook and in the laboratory monitor logbook. If the secondary container is not contaminated, then the packing material and box may be discarded in regular trash after all radioactive material labels have been removed.

5. The radioactive material log is examined to ensure that the quantity received amount coincides with the amount actually received. The row containing this information is dated and initialed. If any discrepancies are present, contact the Principal Investigator immediately.

G. Storage of ^{3}H-NAD^{+}

The ^{3}H-NAD^{+} is stored in a secured or locked system, such as a Revco freezer equipped with a lock, following procedures set forth in Battelle SOP H/SP III-003. The radioactive material vial is, at all times, secondarily contained when the material is outside the fume hood. Both primary and secondary containers, as well as the door to the storage site and immediate area of storage must be conspicuously labeled indicating the presence of radioactive material, isotope, quantity, and chemical nature and form. The Radiation Safety Office is responsible for initially placing the appropriate radioactive labels in the storage facility as well as for removing these labels at the end of the operation.

H. PADPRP System

1. A full-thickness skin specimen, a human skin equivalent system, or cell-based system are the sources of PADPRP to be determined. The tissue and cells are either homogenized, sonicated, or treated with detergent to disrupt membrane integrity and allow the permeation of ^{3}H-NAD^{+}. Tissue or cell samples are prepared prior to the addition of ^{3}H-NAD^{+}. The tissue and cell samples, which have been exposed to or contain exempt levels of HD, are handled in accordance with Battelle SOP MREF I-003.

2. All operations are performed in a fully operational and exempt surety material approved fume hood or biological safety cabinet by an individual wearing scrubs, a disposable lab coat, shoe covers, disposable sleeves, safety glasses, and two pairs of

surgical style gloves, one of which must be nitrile. Following sample preparation, ^{3}H-NAD^{+} (maximum activity of 1 µCi per reaction) is added to the sample. After a room temperature incubation, ice-cold TCA is added to no more than a 10 percent final concentration and the sample held on ice for 10 minutes. The TCA-treated sample is filtered using a Millipore vacuum manifold which contains an inline charcoal filter situated between the manifold and the vacuum pump. The filter membrane is washed to decrease non-specific ^{3}H-NAD^{+} binding and the filter is then carefully removed from the manifold and placed into a scintillation vial using a pair of forceps.

I. Laboratory Monitoring for ^{3}H-NAD^{+} Contamination

1. Laboratory monitoring is performed using smear test which proceeds following Battelle SOP H/SP III-004.

2. Monitoring of the work area is performed following the completion of an assay or at the end of the work day.

3. Smears are taken by an individual appropriately garbed donning two pair of surgical type gloves. Smear should be taken from the areas which are most likely to be contaminated; such as the hood floor, hood wall, hood sash handles and lip, and door handles.

4. All equipment and supplies used in the process must be identified by radiolabel tape. The materials must be maintained in the hood on spill trays until appropriate decontamination steps have been taken and smear monitoring demonstrates that no radiolabel contamination is present. Radiation Safety Services must perform the final smear test to prove the equipment non-contaminated as well as remove the radiolabel from the equipment. Alternatively, the material may be double-bagged, sealed, conspicuously labeled as being radioactive indicating the isotope and level of activity, then removed from the hood.

J. Disposal of ^{3}H-NAD^{+}

Radioactive waste must be segregated by isotope into solid waste, liquid waste - water only, liquid waste - solvents, liquid waste - DeMinimis, liquid waste - biological, and liquid waste - mixed. Any material exposed to exempt level of HD, must first be appropriately decontaminated following Battelle SOP MREF I-003. Solid waste which had been decontaminated, must be separated from the decontamination liquid then subjected to proof-of-decontamination prior to disposal as radioactive waste.

1. Solid Waste - All waste is placed in a sealed 4-mm polyethylene bag. Any sharps or pipets which may puncture the bag are first boxed and the box is placed in the bag. The bag will not be filled by more than three-fourths before sealing by twisting and taping. The bag is placed inside a second bag of similar specifications at the hood face and sealed. Care must be taken to ensure that radioactivity is not transferred to the second bag. This is accomplished by a person holding the second bag wearing clean (fresh) gloves and the other person carefully placing the first bag in the second without touching it. If the waste must be submitted for proof-of-decontamination, then the waste is contained by two additional 4-mm polyethylene bags prior to disposal as radioactive waste.

2. Liquid Waste - All liquid waste is kept in a one-quart to five-gallon plastic bottle equipped with a screw cap. Solvent waste is kept in a container that will not corrode. The lid is tightly secured except when waste is being added.

   a. Water Only - May not contain any type of detectable organic or inorganic material.

   b. Solvents - Includes, but is not limited to, solvents and mixtures of acetone, toluene, xylene, methanol, and scintillation cocktail that does not meet the definition of DeMinimis.

   c. Biological - Includes, but is not limited to tissue extracts which have the potential to decompose or putrefy.

3. DeMinimis Waste - Liquid scintillation cocktail, in which the activity is no greater than 0.05 μCi (111,000 dpm) per mL, is considered DeMinimis. All liquid waste is kept in a one-quart to five-gallon plastic bottle equipped with a screw cap. The waste is handled according to Battelle SOP H/SP III-005. The counts of a given tray are examined and if the highest count does not exceed 111,000 dpm, then the tray may be considered DeMinimis. If the waste has been determined to be DeMinimis, then a DeMinimis Verification Form (Attachment 4) is completed and submitted to the Radiation Safety Office. Once approved, the inventory list is removed from the De Minimis waste and attached to the Verification Form. Radiolabel signs and the material are disposed of following Battelle SOP H/SP III-005.

4. All waste is stored in a secured and appropriately labeled location as designated on the RMA. A running record of the waste generated, isotope, microcurie quantity, physical form, non-radioactive constituents, date, and individual's initials is

kept with each type of waste. The Environmental Safety and Health Department which have regularly scheduled pickups are notified of disposal. For waste which cannot be considered DeMinimis, the running inventory amount is totalled and the information transferred to a Radioactive Waste Label (B-1012). Upon pickup, the running inventory list is clearly marked with the date of waste pickup, Facility Manager's initials (who is in charge of the pickup), and signature of individual picking up the waste.

K. Decontamination, Emergency Procedures, and First Aid Procedures

1. Decontamination of HD-exposed samples proceeds following the procedures outlined in Battelle SOP MREF I-003.

2. Action levels for the results of radiation surveys are as follows:

   a. If equal to or over 100 dpm per 100 sq.cm. in an unrestricted area, then restrict access and immediately decontaminate the area. Re-survey to verify decontamination. The area can only be released from restricted control by the Radiation Safety Officer.

   b. If equal to or over 100 dpm per 100 sq.cm. in a restricted area, post area of contamination and then decontaminate at earliest convenience possible.

   c. If equal to or over 1,000 dpm per 100 sq.cm., contact the Principal Investigator immediately who, in turn, will contact Radiation Safety Services. Decontaminate the area as soon as possible, by the close of the business day.

   d. If equal to or over 10,000 dpm per 100 sq.cm., stop work immediately, post as a contaminated area, contact the Principal Investigator immediately who, in turn, will contact Radiation Safety Services. Radiation Safety Services will perform air samples to determine airborne concentrations. Decontaminate as directed by Radiation Safety Services. Radiation Safety Services will post the post-decontamination survey results prior to resuming work.

3. Surfaces, equipment, etc., contaminated with radiolabel compound are decontaminated with a solution such as Isoclean or Count-Off following procedures outlined in Battelle SOP H/SP III-003. Activity greater that 100 dpm per 100 sq.cm. is the action level requiring decontamination.

4. Radioactive spills are contained and cleaned up following procedures in Battelle SOP H/SP III-003. The spill is first contained using kimwipes or other type of absorbent material. After containment and taking care of any personnel contamination, the Principal Investigator is notified who, in turn, notifies management and the Radiation Safety Office.

5. Personnel contaminated with radiolabel material are to respond in accordance with Battelle SOP H/SP III-003. Contaminated clothing is removed and placed in a waste bag, the affected areas are washed with water and the water collected as being radioactive waste. The Principal Investigator is then notified, who, in turn, notifies management and the Radiation Safety Office. Urine bioassays are collected 3 to 4 hr following personnel exposure following Battelle SOP H/SP III-002.

L. Record Maintenance

1. The location of all smears, smear results, and any action taken for contaminated areas must be maintained in a Laboratory Monitor Logbook using the form shown in Attachment 2.

2. A radioactive material log as shown in Attachment 1 must also be maintained and updated any time that radioactive material is removed.

3. Records of waste material disposition must be maintained for each classification of waste generated and maintained in the running inventory logs kept with the waste.

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ATTACHMENT 1

CUSTODIAN DAILY USE LOG

Date	Serial #	Transferred to: Name/Location	Time Out/INT	Time In/INT

MREF

ATTACHMENT 2

LABORATORY SURVEY REPORT

DATE:	INSTRUMENTATION USED		
TIME:	MODEL	S/N	CAL DUE DATE
SURVEYOR:			
LOCATION:			
PURPOSE OF SURVEY:			

SMEAR RESULTS IN DPM/100CM^2 UNLESS OTHERWISE NOTED. ATTACH LSC PRINTOUT

ACTION LEVELS:	Fixed: >1000 dpm/100 cm^2	**NOTIFY RADIATION SAFETY SERVICES OF ANY LOOSE CONTAMINATION ≥1000 DPM 100 CM^2**
RSS-015	≥0.25 mRem/hr.	
	Loose: ≥100 dpm/100cm^2	

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DRAFT

ATTACHMENT 3
RADIOACTIVE MATERIAL TRANSFER

Instructions:

1. Complete this form for all transfers of radioactive material between the King Avenue and the West Jefferson campuses
2. **Verify the authorization** of the intended receiver with the Radiological Safety Officer (RSO) of the receiving institution **before actual transfer.**
3. All packages transported on public roads **must comply with the shipping regulations** of the U.S. Department of Transportation.
4. **Send appropriate copies of this form to addresses shown.**

Licensee Identification:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201

Transaction: ☐ KA to WJ or ☐ WJ to KA Date: _____

Material Description:

Nuclide: _____ Activity: _____ millicuries

Form (compound): _____

Purchase Order #: _____ RMA No. _____

User Identification (type or print legibly):

Responsible User: Authorized User:

_____

_____

Sender sign here _____

Receiver sign here and keep copy _____

Send original to RSO: Dennis Clum
Room 1-4-10, (614) 424-7676

ATTACHMENT 4

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LIQUID SCINTILLATION MEDIA DE MINIMIS VERIFICATION FORM

NUCLIDE _____ DATE _____

PROJECT NO. _____ RMA # _____

I. Explanation and Qualification of the Exemption

De minimis classification applies only to liquid scintillation media containing less than 0.05 μCi (microcuries) of H-3 and C-14 per gram of medium. (<111,000 dpm per gram of medium)

1. De minimis materials may be disposed of without regard to their radioactivity.
2. De minimis provisions do not relieve persons from compliance with radioactive accounting regulations or any other regulations governing management of hazardous materials.

II. Verification of Waste as a De Minimis Quantity for Disposal

Sample Selection

1. Each time you count a group of samples, record the highest sample count. Retain a verified copy of the read-out for the highest sample count to-date in the lab survey record book.
2. When the waste container (original case) is full, use the highest sample count rate for your verification calculation. For bulk samples, ensure that the liquid is well mixed and take three samples for determination of activity. Use the highest sample count rate of the three for your verification calculation.

Calculation

Record the dpm for the highest activity vial here: (a) _____ dpm

Divide (a) by 2,220,000 dpm/μCi. Record μCi's here: (b) _____ μCi

Record volume of counting medium in the highest activity vial here: (c) _____ ml

Divide (b) by (c) and record value here: (d) _____ μCi/ml
Note: (d) must be less than 0.05 μCi/ml to be qualified as de minimis.

III. Total Volume and Activity

Record total number of vials in box here (not req. for bulk samples): (e) _____
Note: All vials must contain some volume of counting medium.

Multiply (e) by (c). Record total volume here: (f) _____ ml.
Note: Record total volume for bulk samples here

Multiply (f) by (d). Record total activity here: _____ μCi.

IV. Known Hazard or Pathogen Other Than Radioactivity? yes/no (Circle One)

Note: This includes the scintillation medium and sample.

_____
If yes, list: hazard of pathogen

Submitted by _____ _____
Signature Printed Name

Approved by _____ _____
Radiation Safety Officer Date

APPENDIX C

SUMMARY STATISTICS FOR HD-INDUCED NAD^+ DEPLETION AT 24 HR AS A FUNCTION OF HD CONCENTRATION

TABLE C-1. NAD DEPLETION RESPONSE STUDIES, DESCRIPTIVE STATISTICS BY HD CONCENTRATION AND RUN

HD Conc (μM)	Run Number	N	NAD Conc. Mean (Std. Dev.)	%Depletion Mean (Std. Dev.)
0	1	3	82.13 (3.35)	0.00 (4.08)
	2	4	88.28 (15.46)	0.00 (17.51)
	3	4	147.18 (7.44)	0.00 (5.05)
	4	4	168.43 (3.16)	0.00 (1.88)
	5	4	224.65 (14.96)	0.00 (6.66)
14	3	4	140.47 (12.62)	4.55 (8.57)
	4	4	163.57 (8.85)	2.88 (5.26)
	5	4	220.23 (5.35)	1.97 (2.38)
28	1	3	80.23 (8.13)	2.31 (9.90)
	2	4	90.07 (5.79)	-2.04 (6.56)
	3	4	134.32 (14.93)	8.73 (10.15)
	4	4	158.73 (5.93)	5.76 (3.52)
	5	4	215.47 (7.70)	4.08 (3.43)
56	1	3	68.73 (5.16)	16.31 (6.28)
	2	4	61.58 (6.29)	30.25 (7.13)
	3	4	126.57 (7.33)	14.00 (4.98)
	4	4	136.37 (7.72)	19.03 (4.58)
	5	4	186.93 (5.19)	16.79 (2.31)
113	1	3	48.80 (15.68)	40.58 (19.09)
	2	4	35.97 (12.03)	59.25 (13.63)
	3	4	57.70 (10.62)	60.79 (7.21)
	4	4	60.40 (4.14)	64.14 (2.46)
	5	4	94.55 (2.66)	57.91 (1.18)
144	1	3	25.97 (2.45)	68.38 (2.98)
	2	4	25.10 (7.34)	71.57 (8.32)
	3	4	49.40 (3.46)	66.43 (2.35)
	4	4	51.42 (2.92)	69.47 (1.73)
	5	4	66.82 (3.22)	70.25 (1.44)
180	3	4	36.78 (4.04)	75.01 (2.74)
	4	4	42.58 (6.22)	74.72 (3.69)
	5	4	52.38 (2.09)	76.69 (0.93)

TABLE C-1.
(Continued)

HD Conc (μM)	Run Number	N	NAD Conc. Mean (Std. Dev.)	%Depletion Mean (Std. Dev.)
225	1	3	21.13 (2.10)	74.27 (2.55)
	2	4	21.75 (2.78)	75.36 (3.15)
	3	4	26.15 (4.59)	82.23 (3.12)
450	1	3	9.30 (0.79)	88.68 (0.97)
	2	4	12.43 (2.20)	85.92 (2.50)
	3	4	9.28 (6.68)	93.70 (4.54)
	4	4	7.92 (1.61)	95.29 (0.96)
	5	4	0.00 (0.00)	100.00 (0.00)

APPENDIX D

SUMMARY STATISTICS FOR HD-INDUCED CYTOTOXICITY AT 24 HR AS A FUNCTION OF HD CONCENTRATION

TABLE D-1. DESCRIPTIVE STATISTICS FOR 24-WELL CELL VIABILITY RESPONSE BY HD CONCENTRATION AND EXPERIMENT DATE

Experiment Date	Run #	HD Conc. (μM)	Propidium Iodide		
			Percent Viability		Percent Response
			N	Mean (S.D.)	Mean (S.D.)
05/20/93	1	0.0	3	89.9 (1.1)	0.0 (1.3)
		42.5	3	82.4 (2.6)	8.4 (2.9)
		85.0	3	64.3 (4.9)	28.5 (5.4)
		127.5	3	33.1 (8.5)	63.2 (9.5)
		170.0	3	15.1 (1.5)	83.2 (1.7)
		255.0	-	-	-
05/28/93	2	0.0	4	75.0 (10.5)	0.0 (14.0)
		42.5	4	40.3 (8.9)	46.3 (11.9)
		85.0	4	26.7 (4.2)	64.4 (5.6)
		127.5	4	13.4 (3.0)	82.1 (4.0)
		170.0	4	11.6 (3.6)	84.6 (4.8)
		255.0	4	10.7 (2.9)	85.7 (3.8)
06/10/93	3	0.0	4	75.9 (6.5)	0.0 (8.5)
		21.3	4	83.7 (3.3)	-10.2 (4.4)
		42.5	4	78.0 (9.6)	-2.8 (12.6)
		85.0	4	78.8 (5.3)	-3.8 (7.0)
		127.5	4	43.8 (6.9)	42.3 (9.1)
		170.0	4	23.3 (4.5)	69.3 (6.0)
07/24/93	4	0.0	4	90.0 (3.9)	0.0 (4.3)
		50.0	4	81.0 (3.4)	10.0 (3.8)
		100.0	4	65.4 (4.0)	27.3 (4.4)
		150.0	4	38.0 (2.9)	57.8 (3.3)
		200.0	4	25.2 (3.6)	72.0 (4.0)
		300.0	4	20.2 (1.9)	77.5 (2.1)

APPENDIX E

GRAPHS OF NAD^+ DATA FOR NIACINAMIDE-PRETREATED, HD-EXPOSED CULTURES

FIGURE E-1.

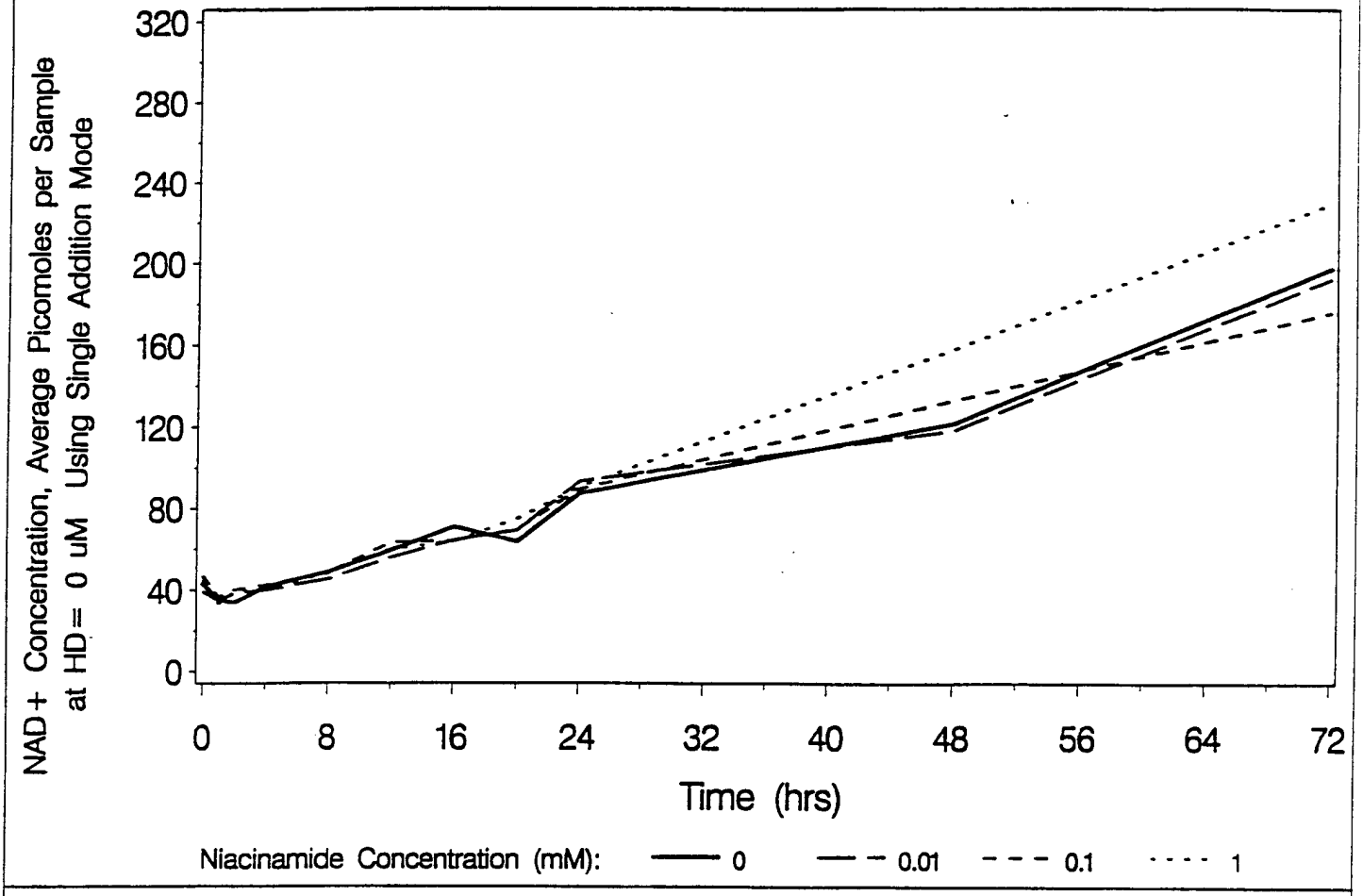


FIGURE E-2.

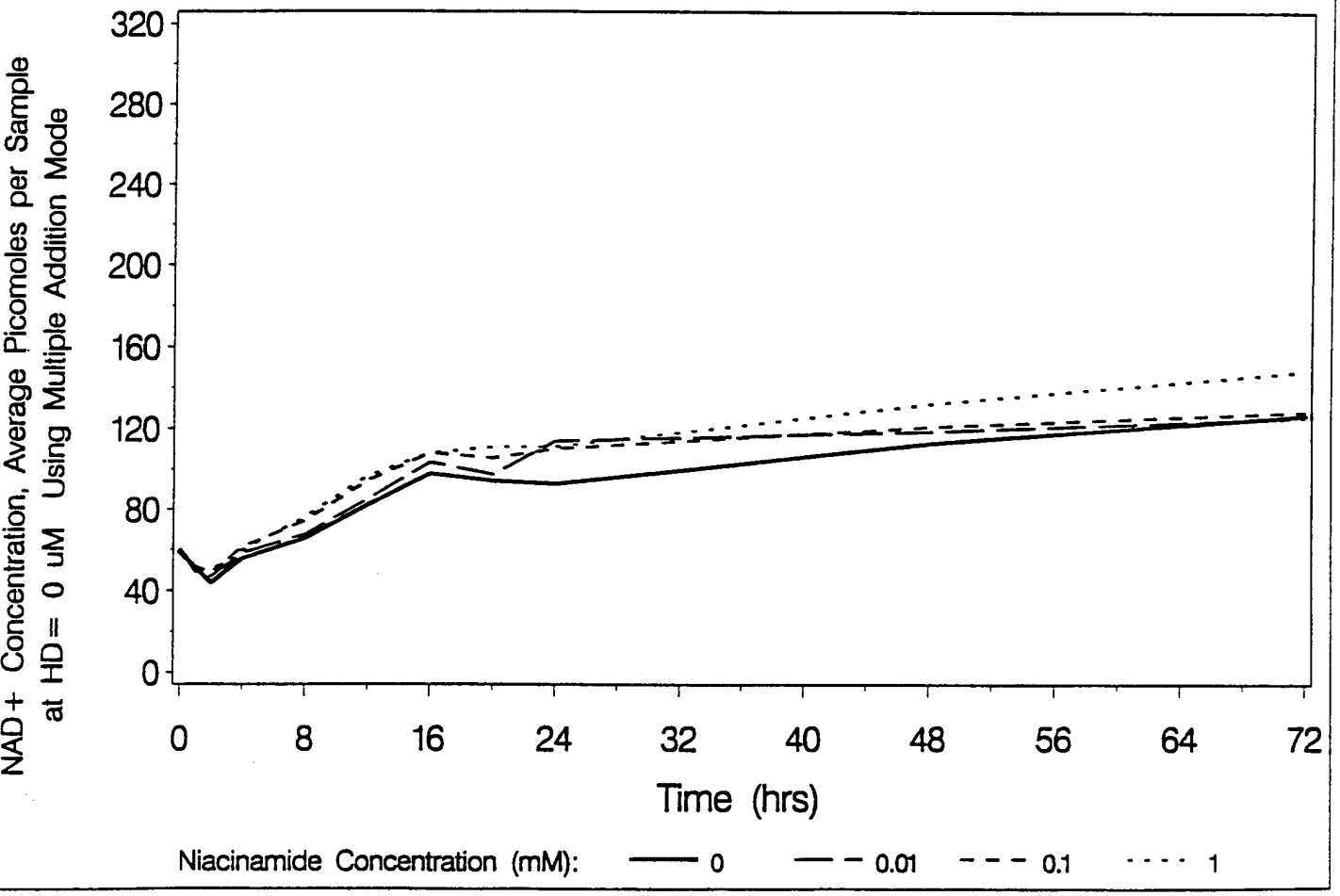


FIGURE E-3.

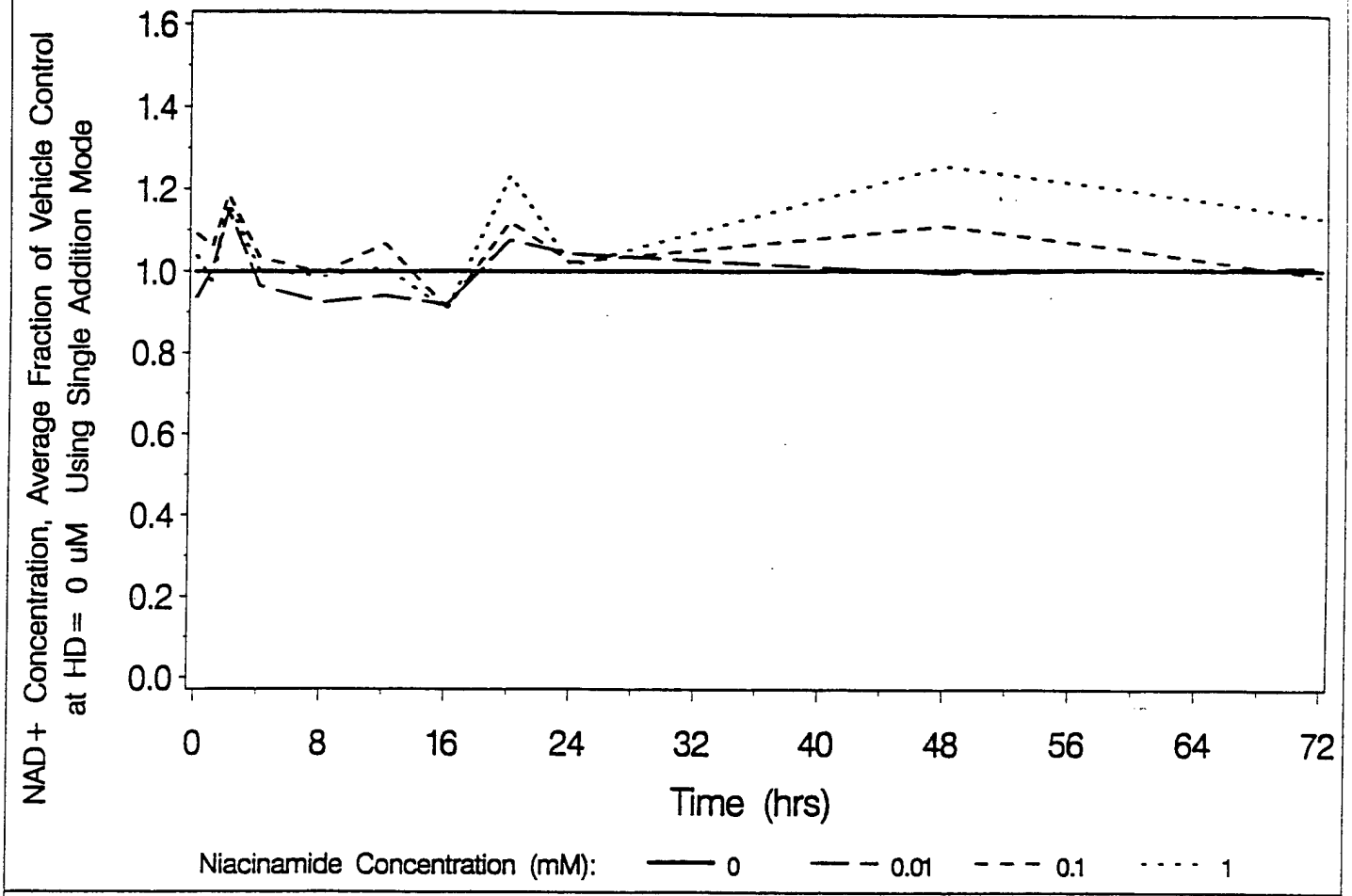


FIGURE E-4.

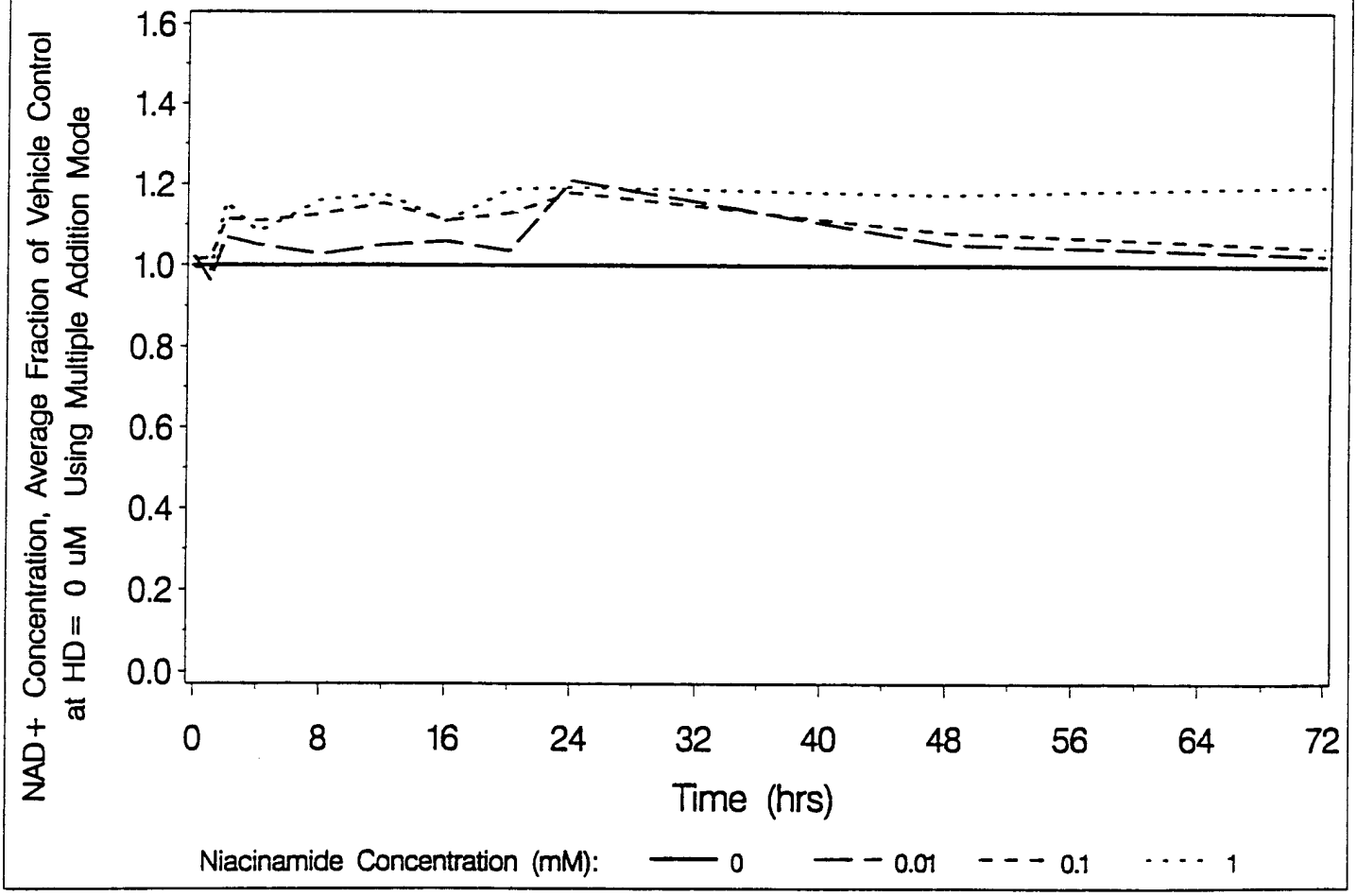


FIGURE E-5.

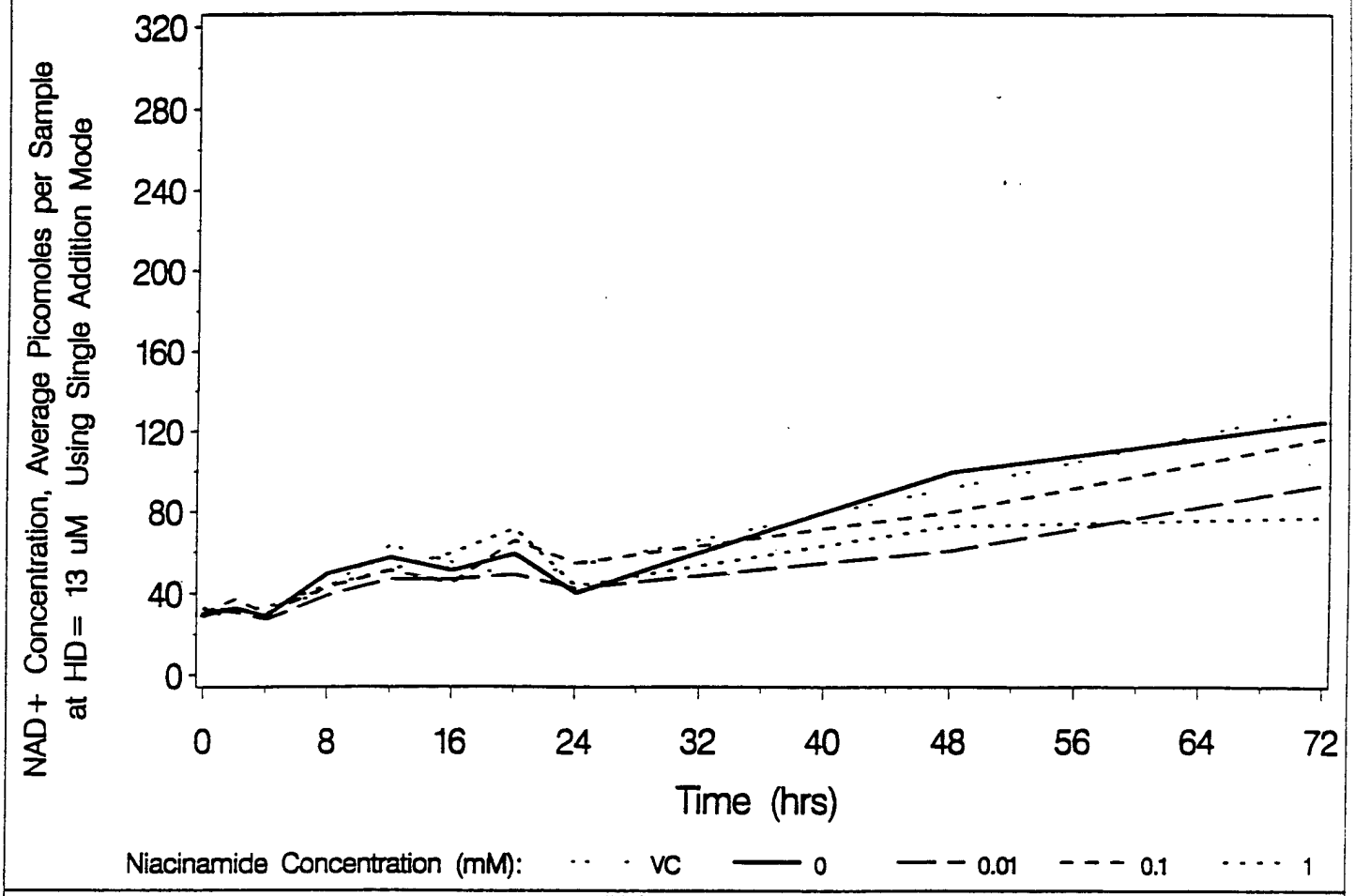


FIGURE E-6.

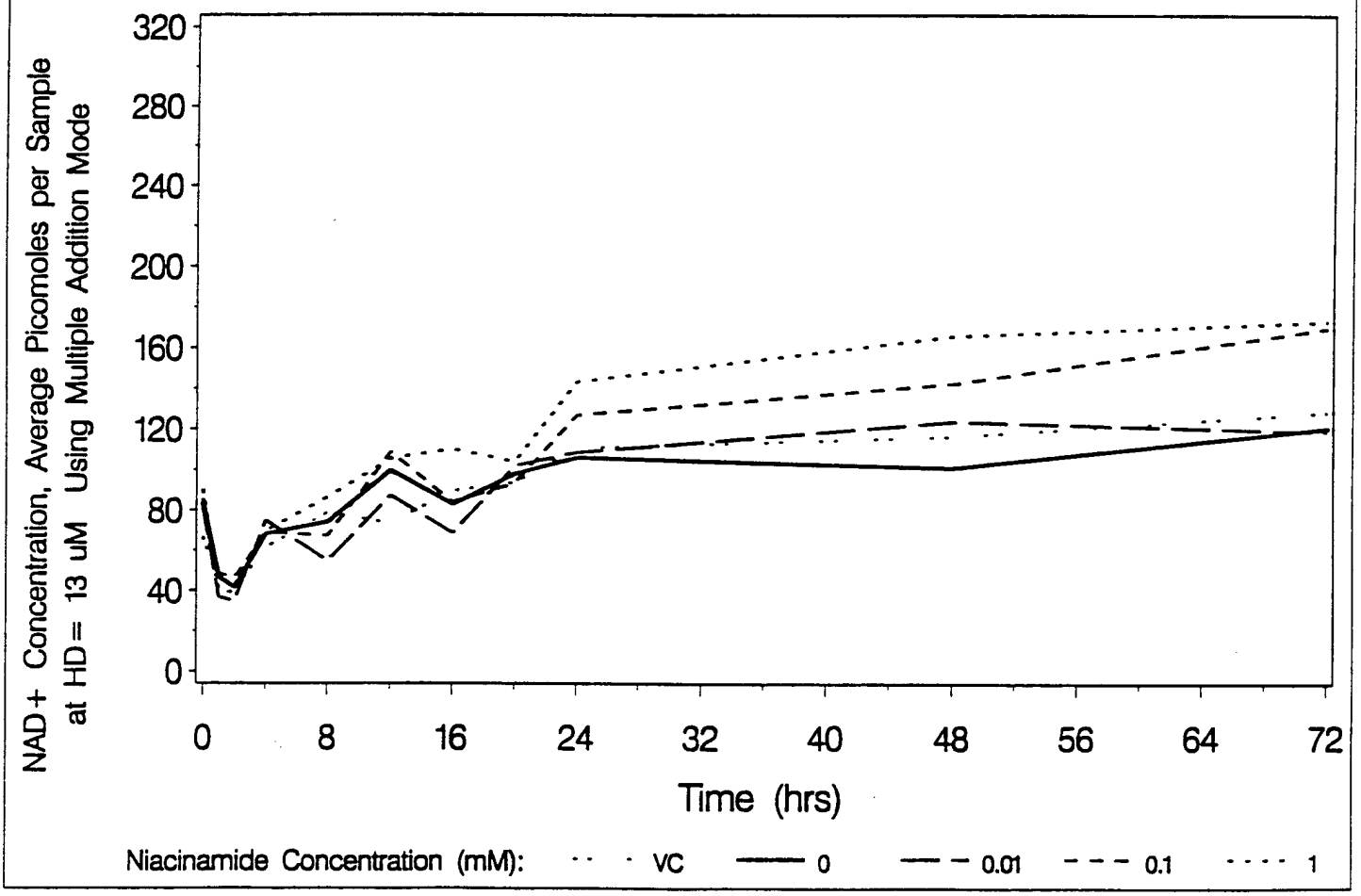


FIGURE E-7.

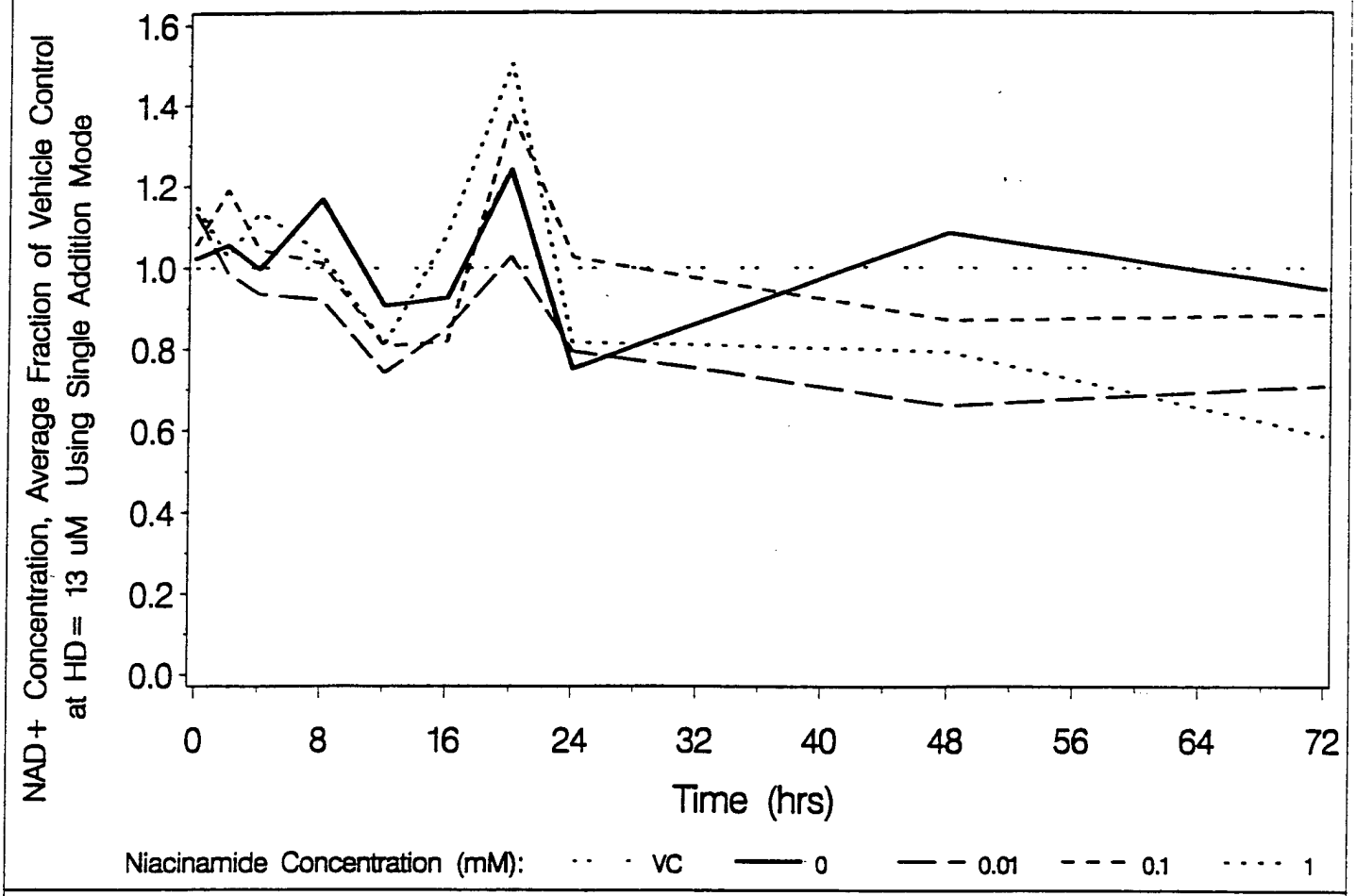


FIGURE E-8.

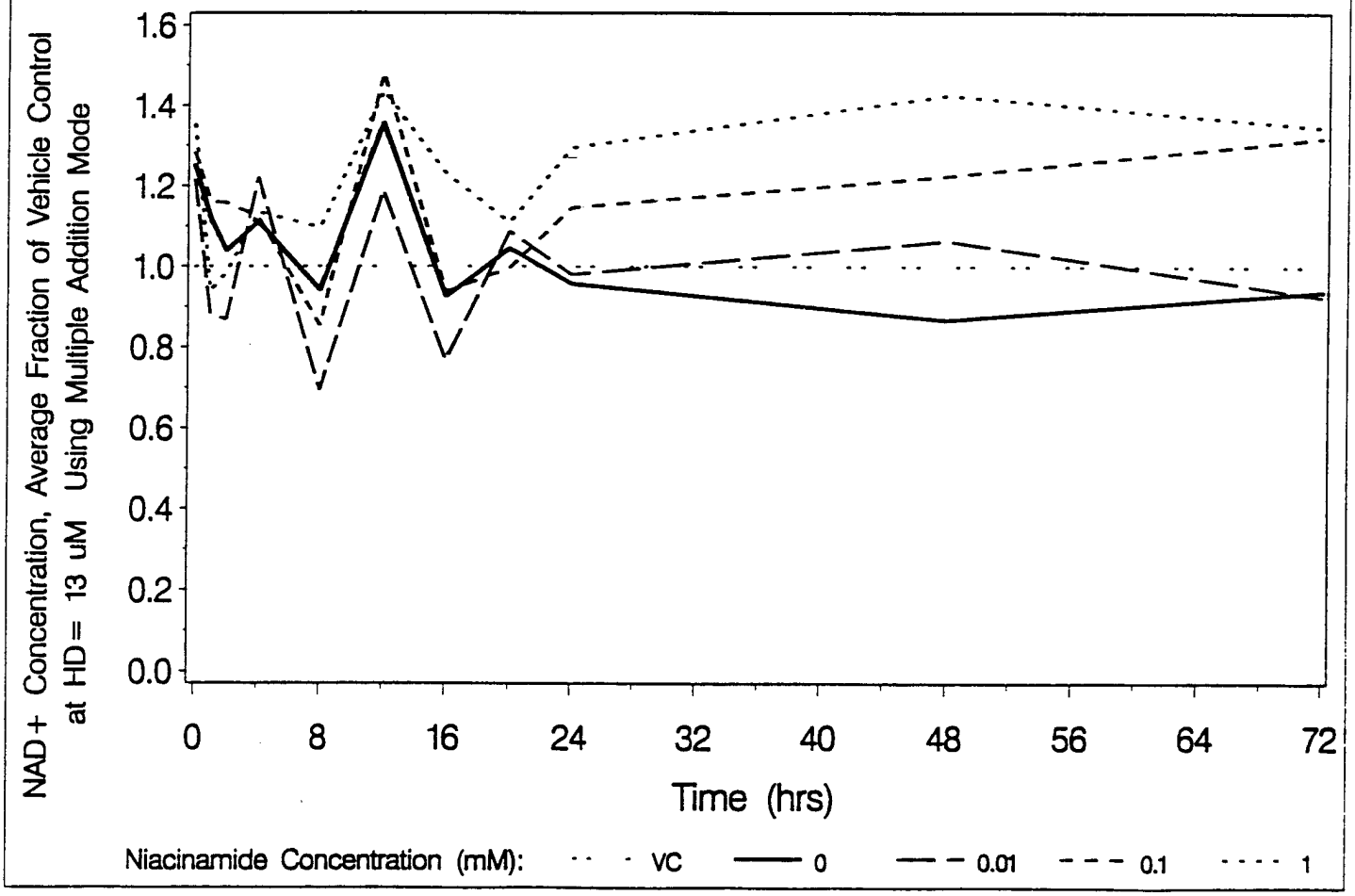


FIGURE E-9.

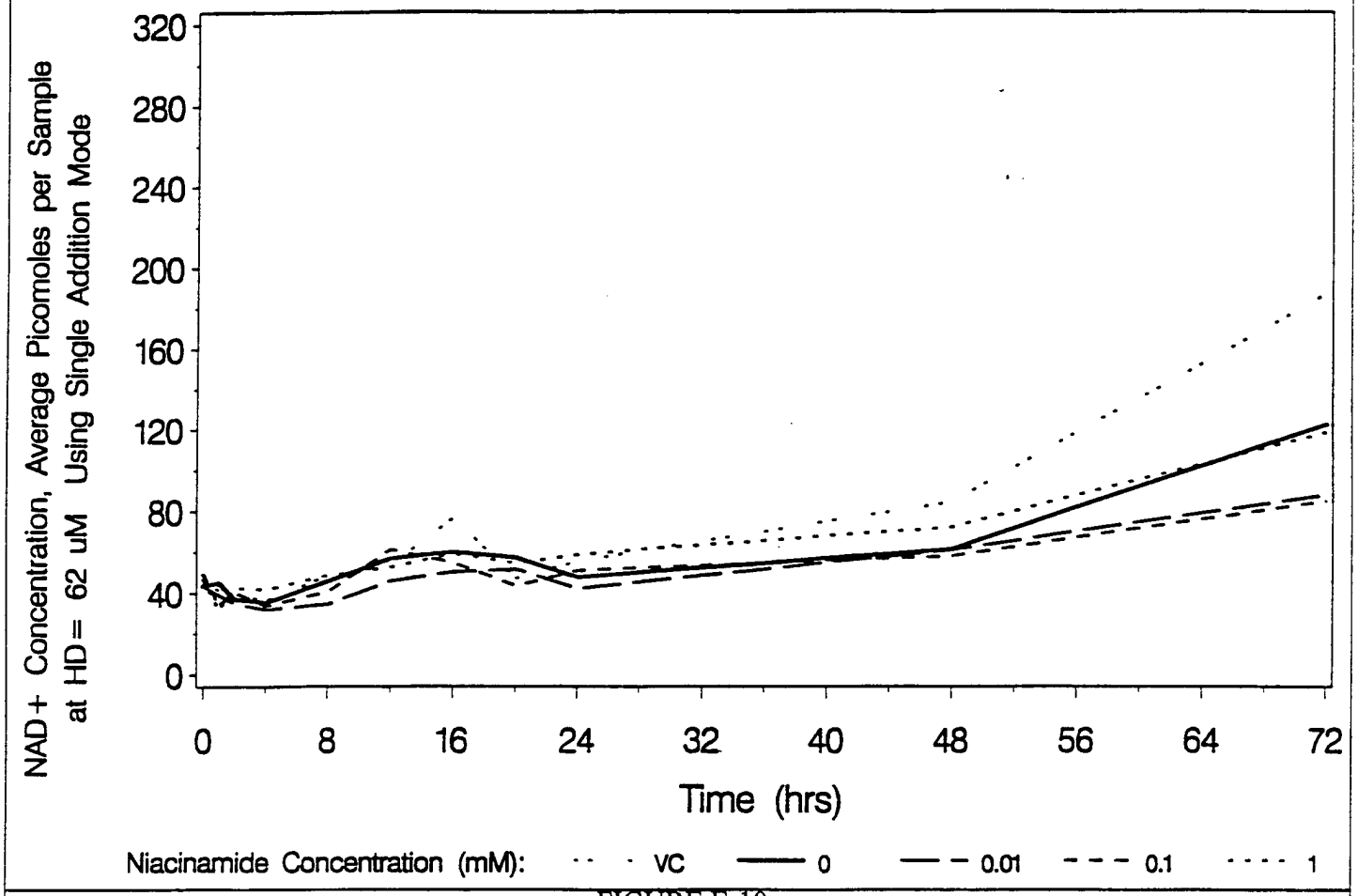


FIGURE E-10.

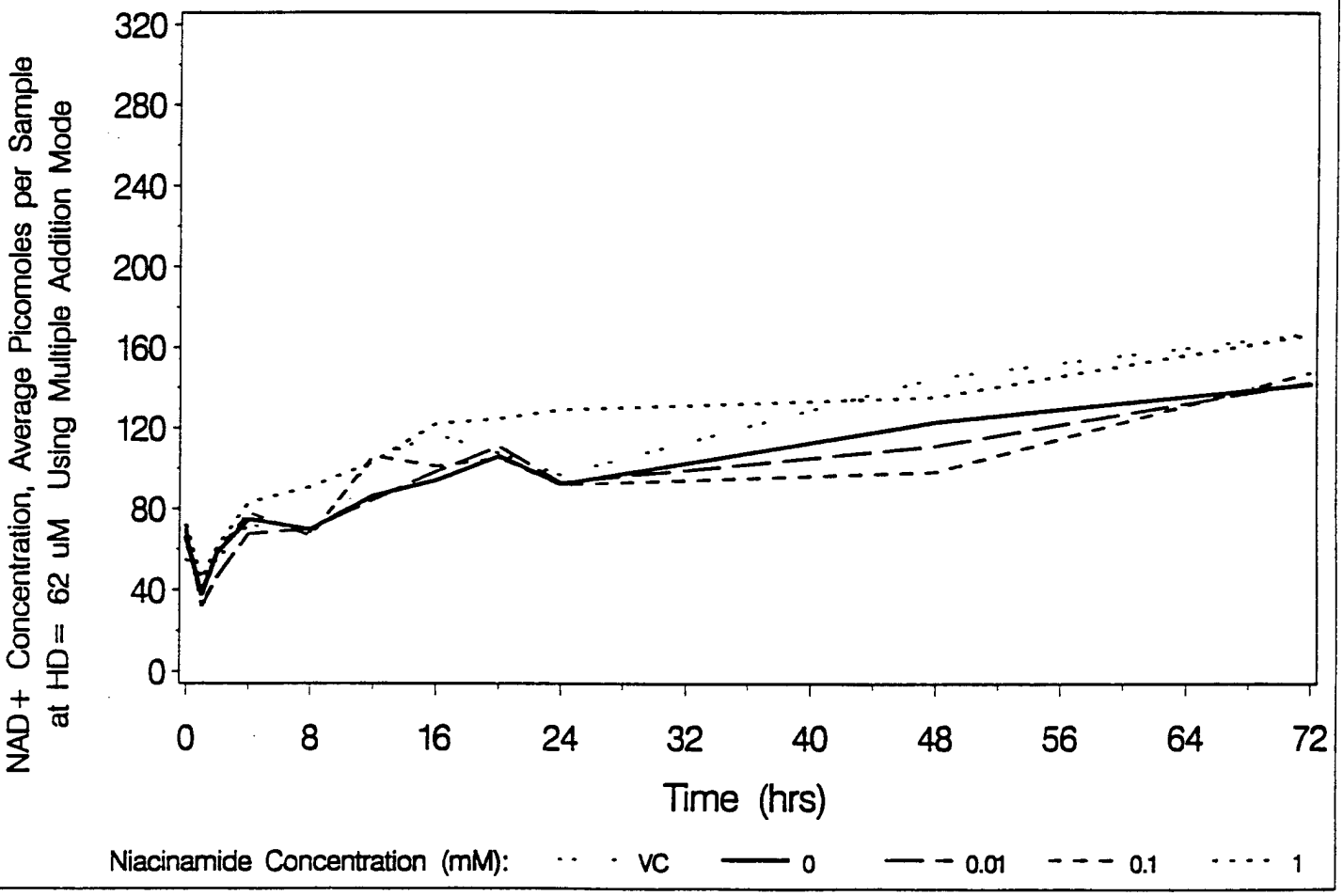


FIGURE E-11.

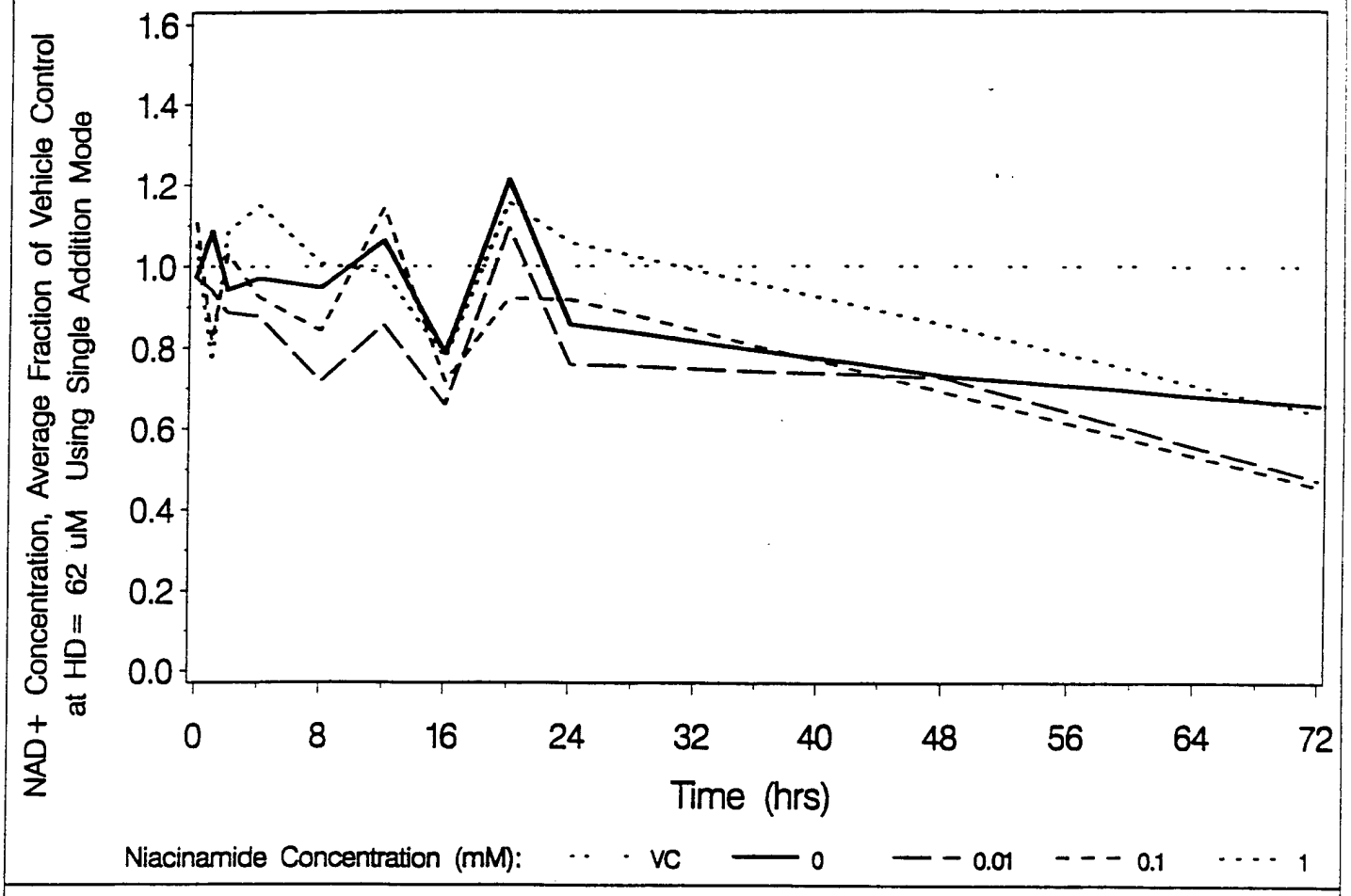


FIGURE E-12.

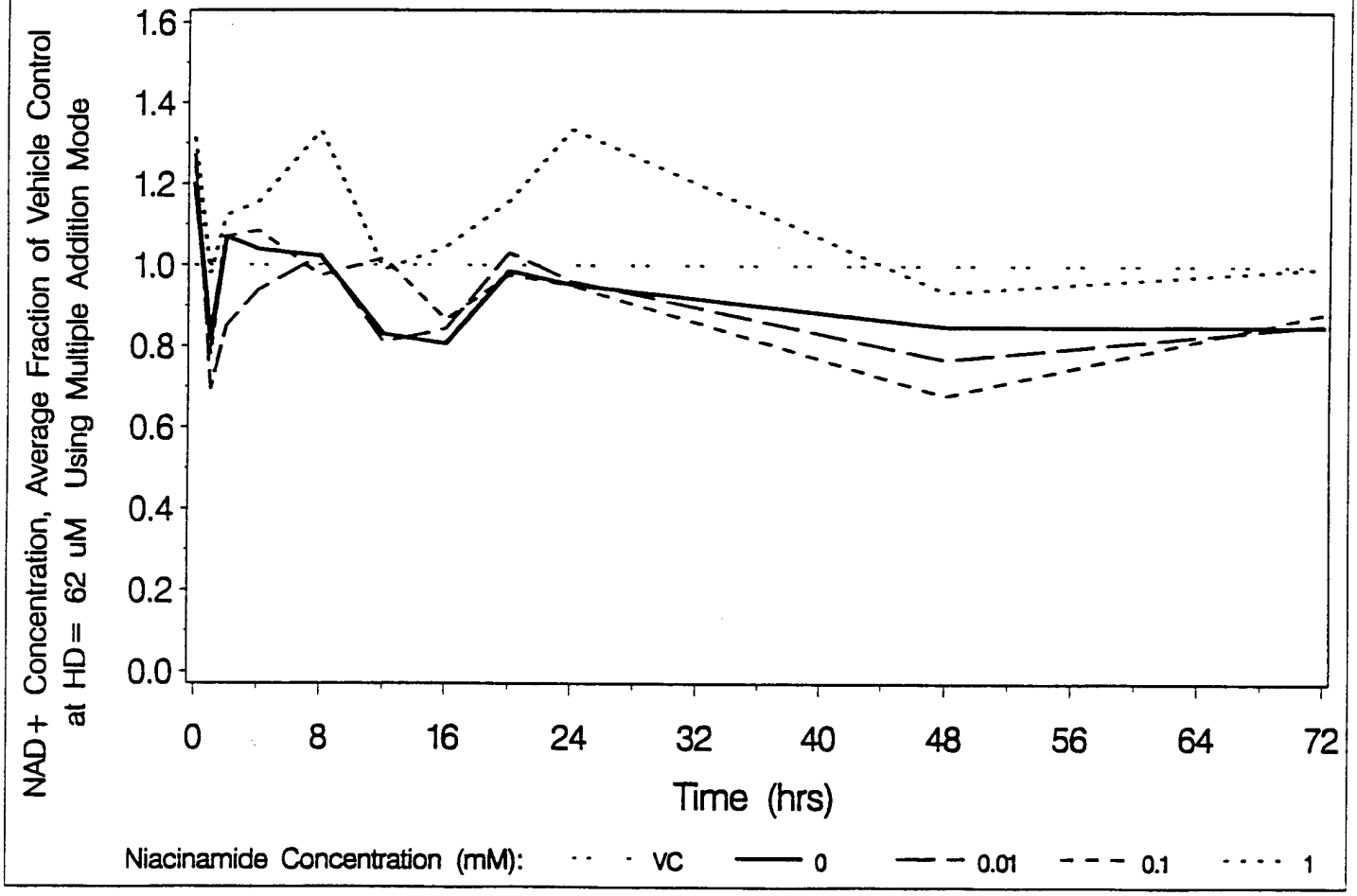


FIGURE E-13.

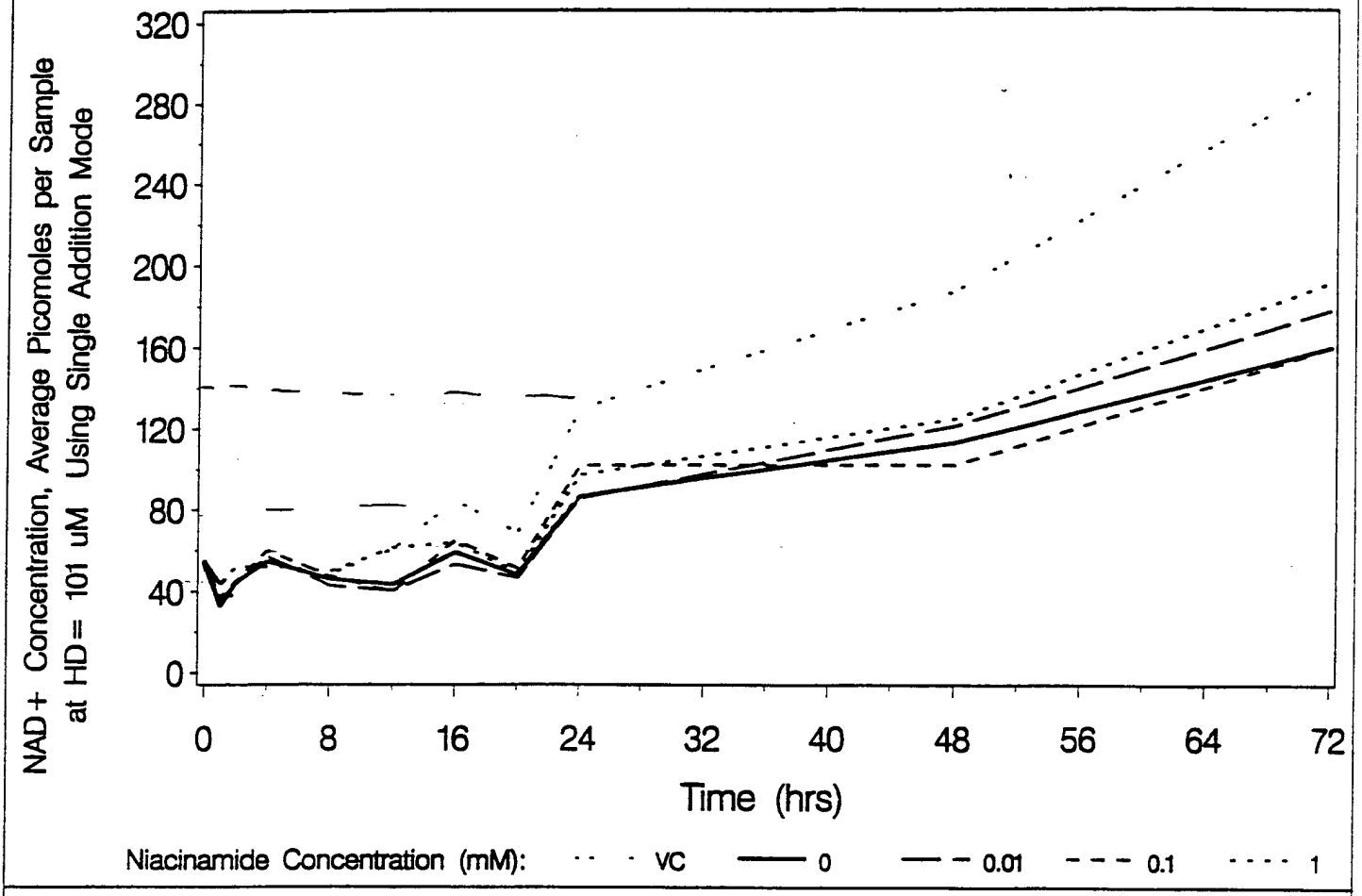


FIGURE E-14.

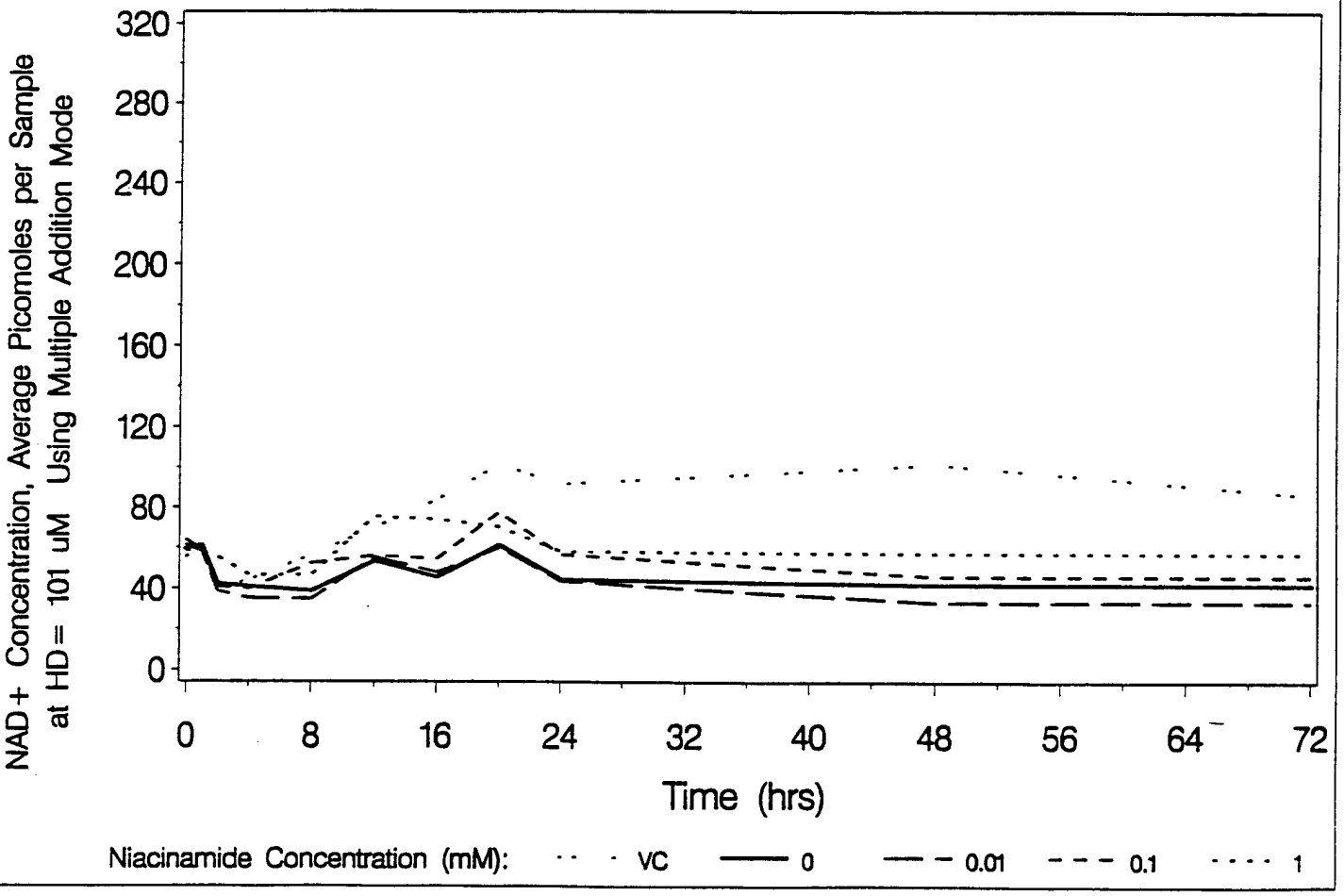


FIGURE E-15.

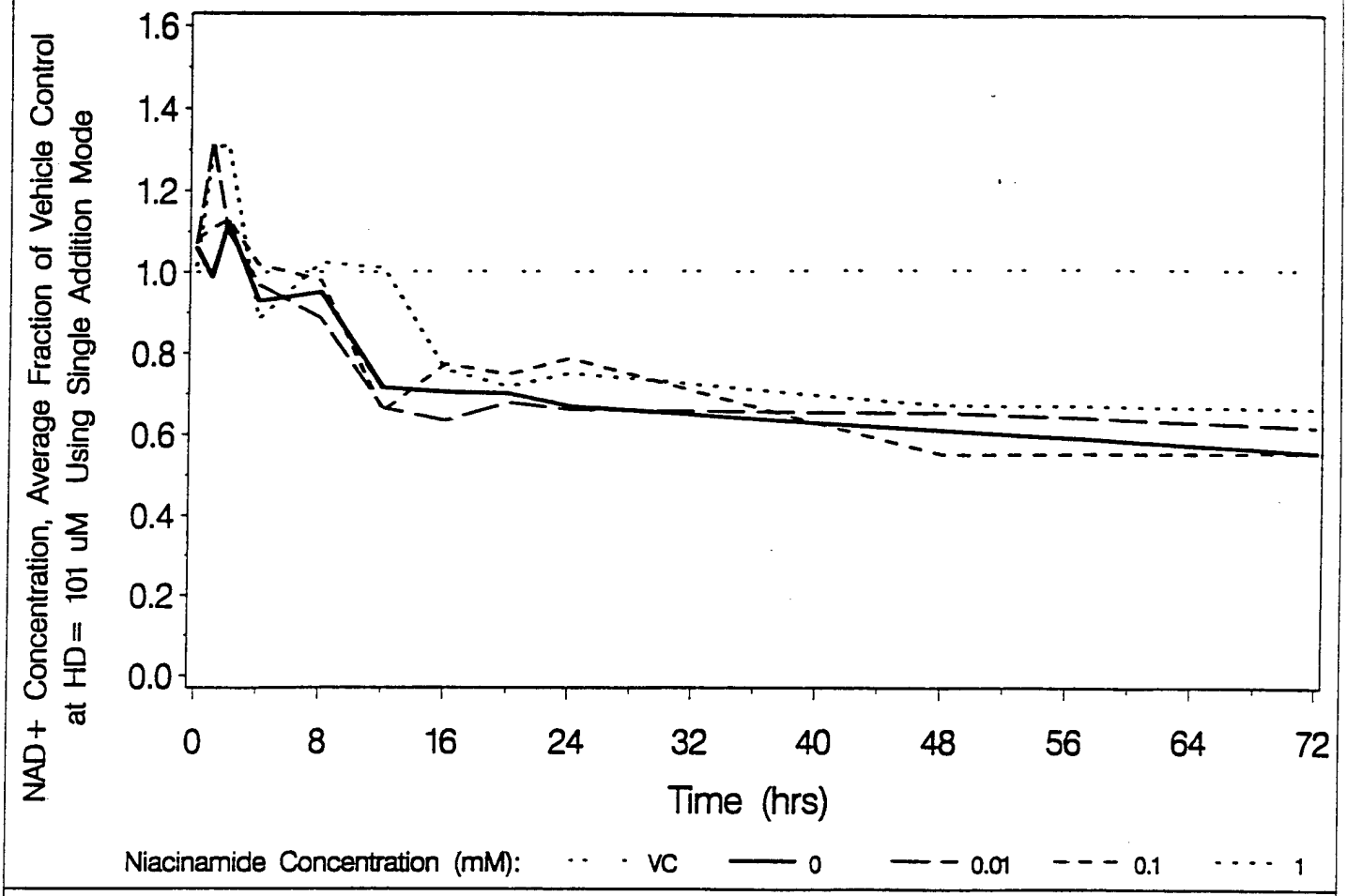


FIGURE E-16.

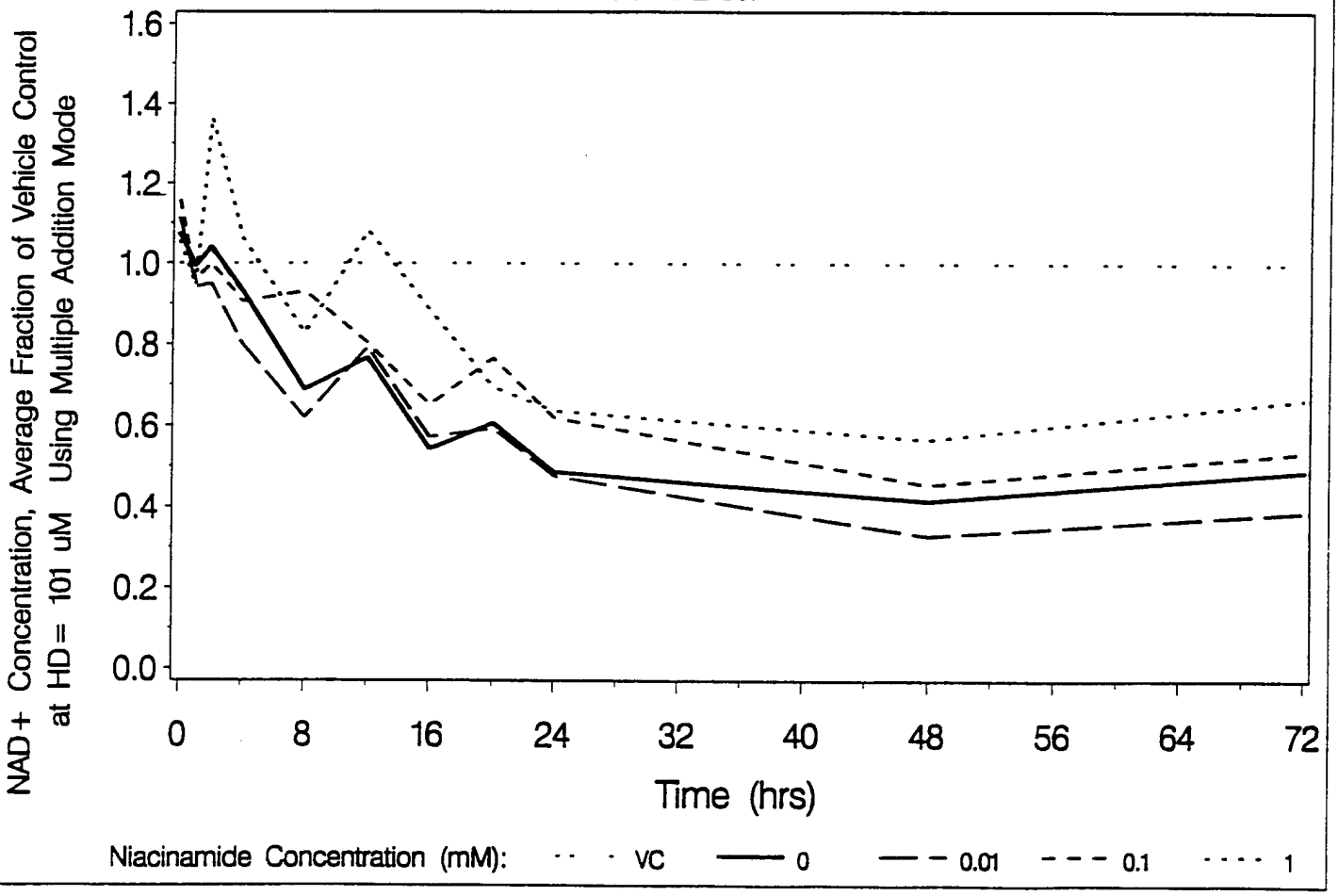


FIGURE E-17.

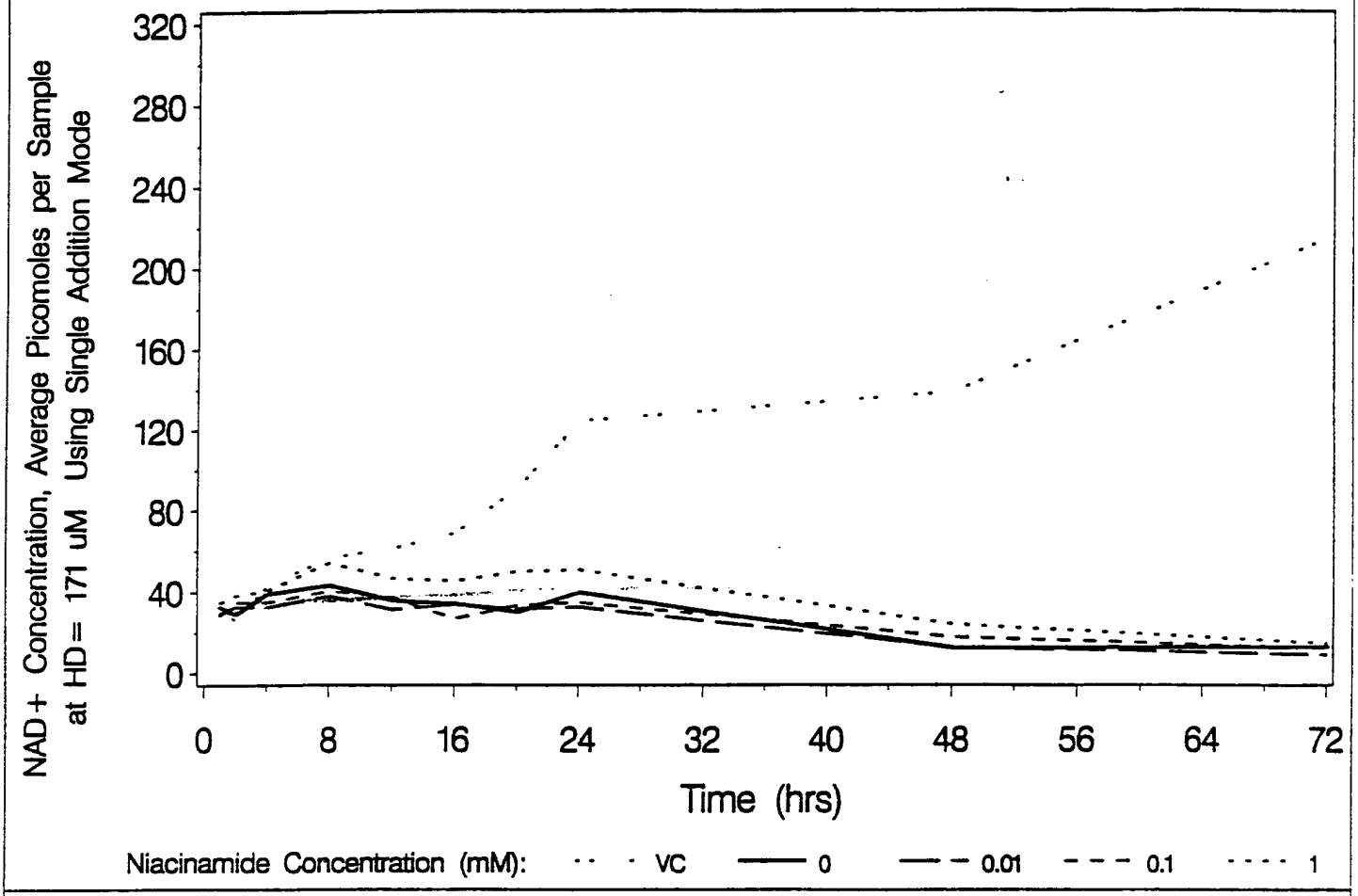


FIGURE E-18.

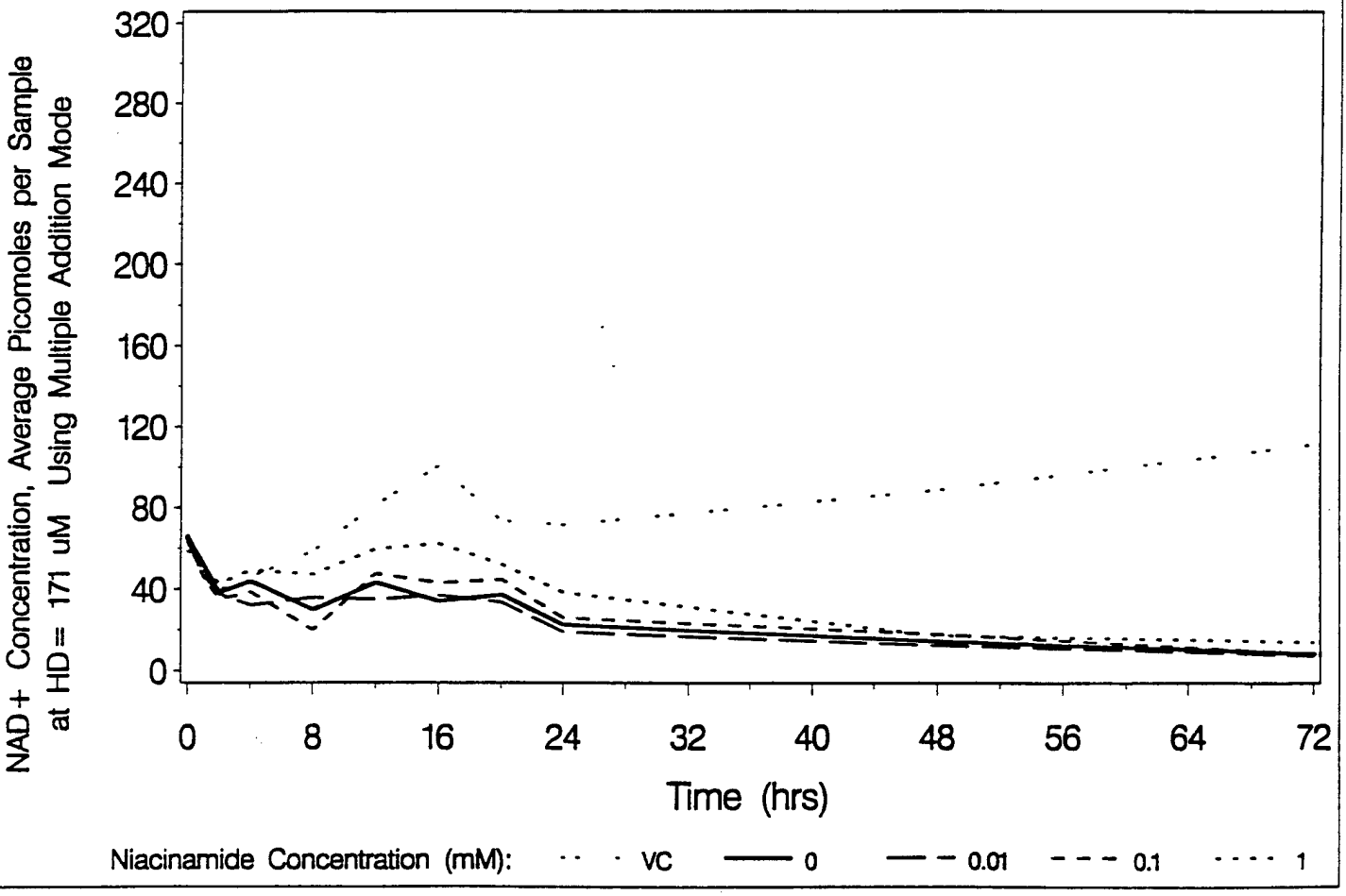


FIGURE E-19.

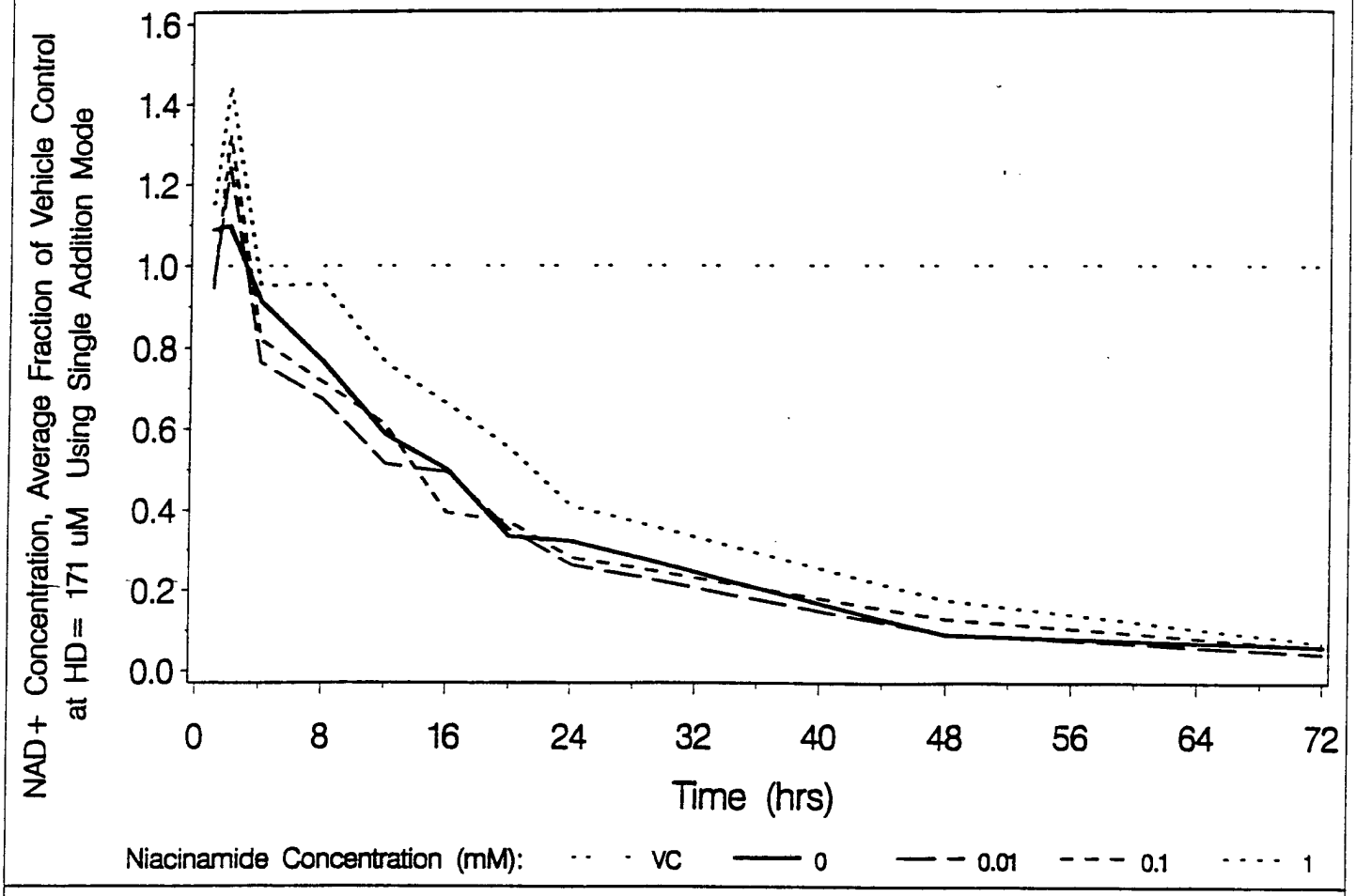
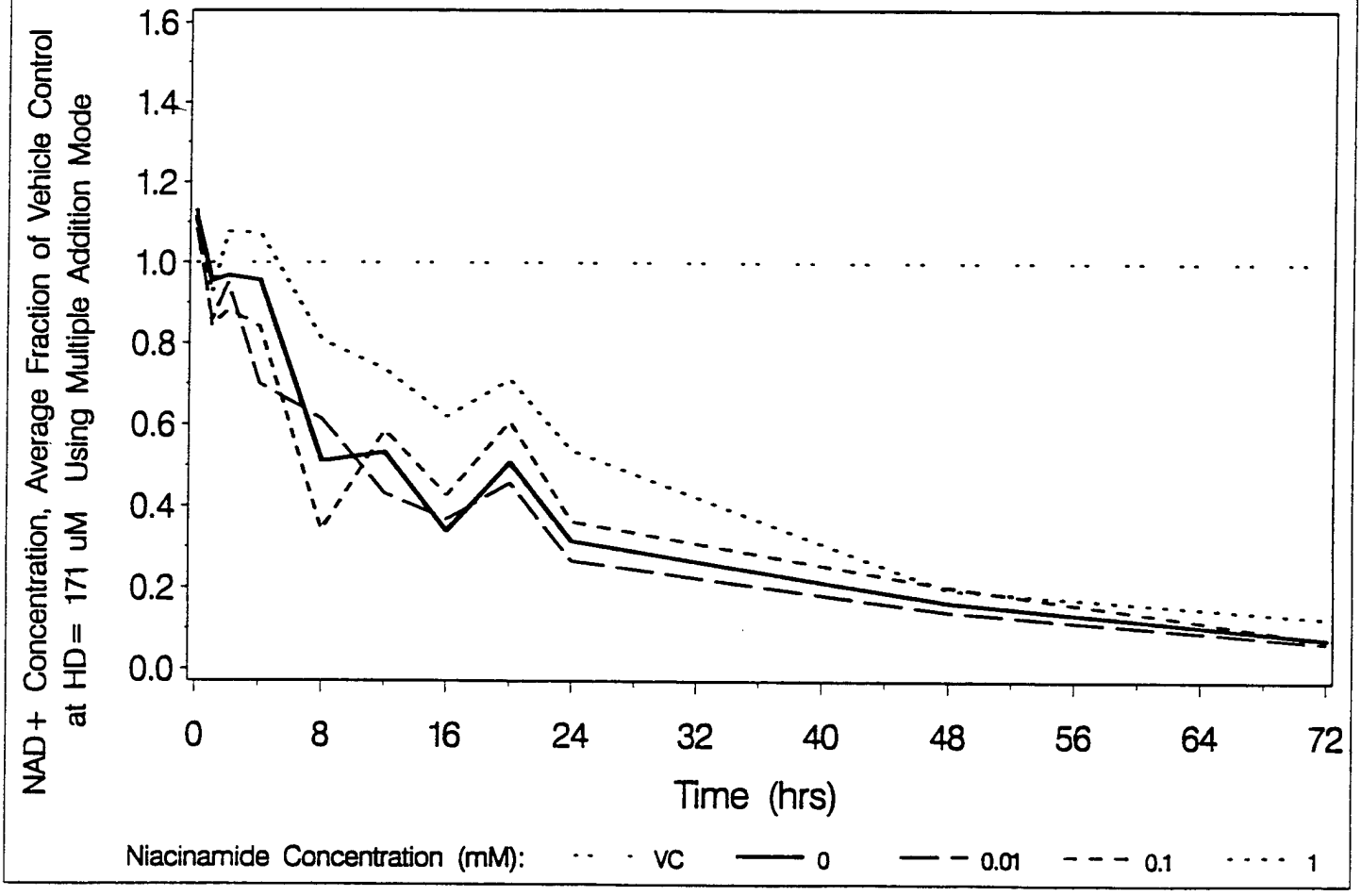


FIGURE E-20.



APPENDIX F

GRAPHS OF NAD^+ DATA FOR NIACIN-PRETREATED, HD-EXPOSED CULTURES

FIGURE F-1.

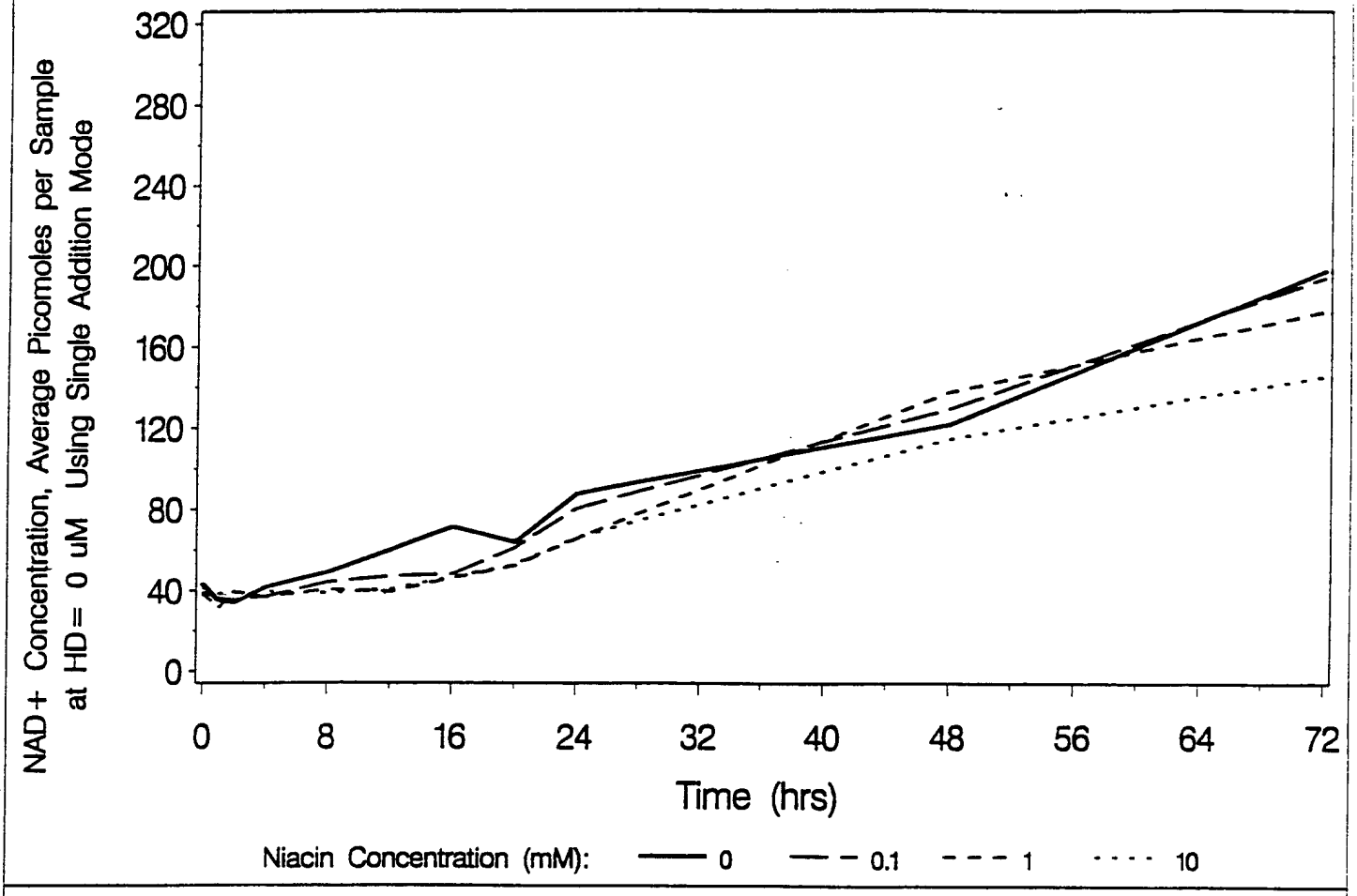


FIGURE F-2.

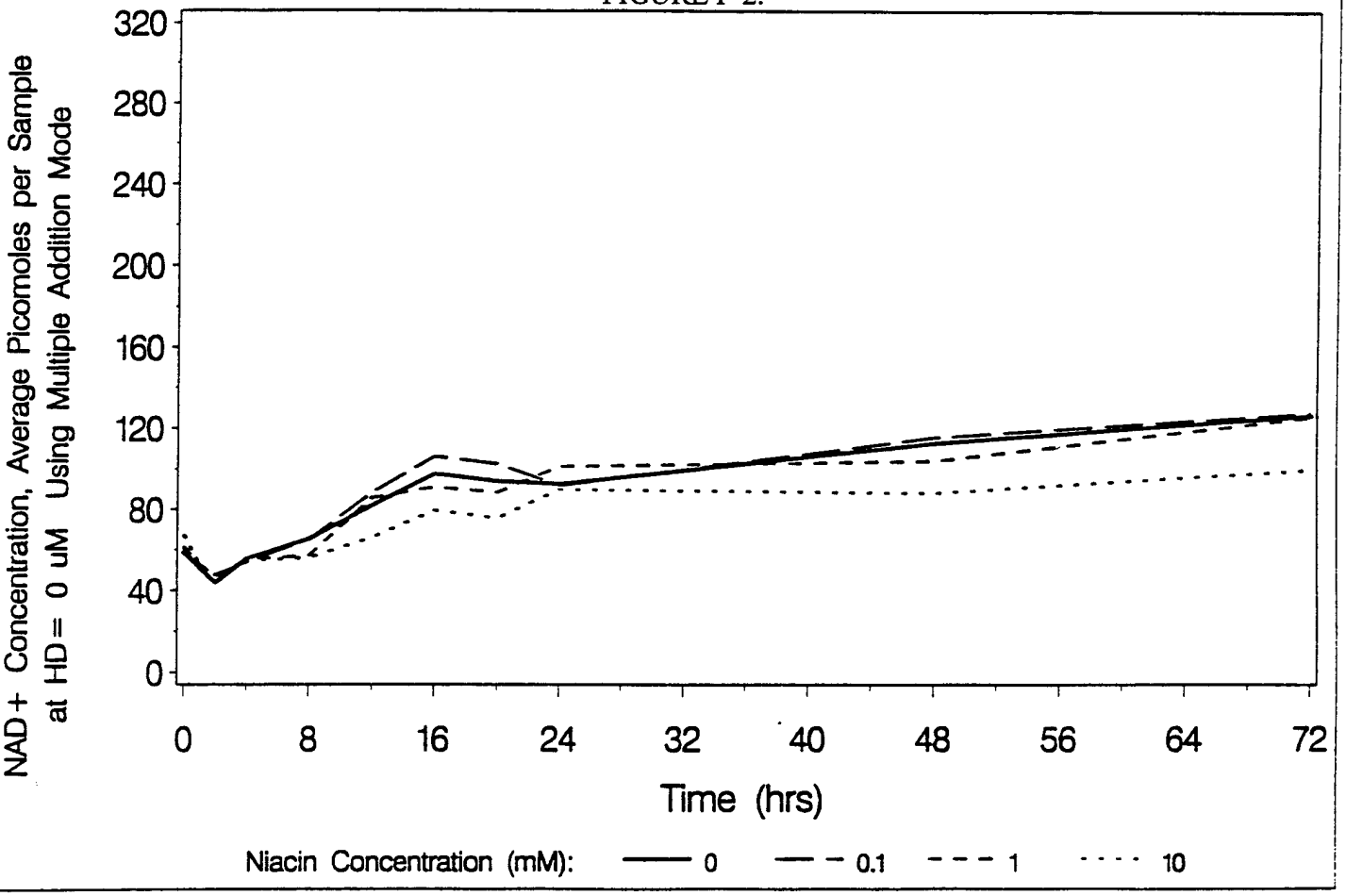


FIGURE F-3.

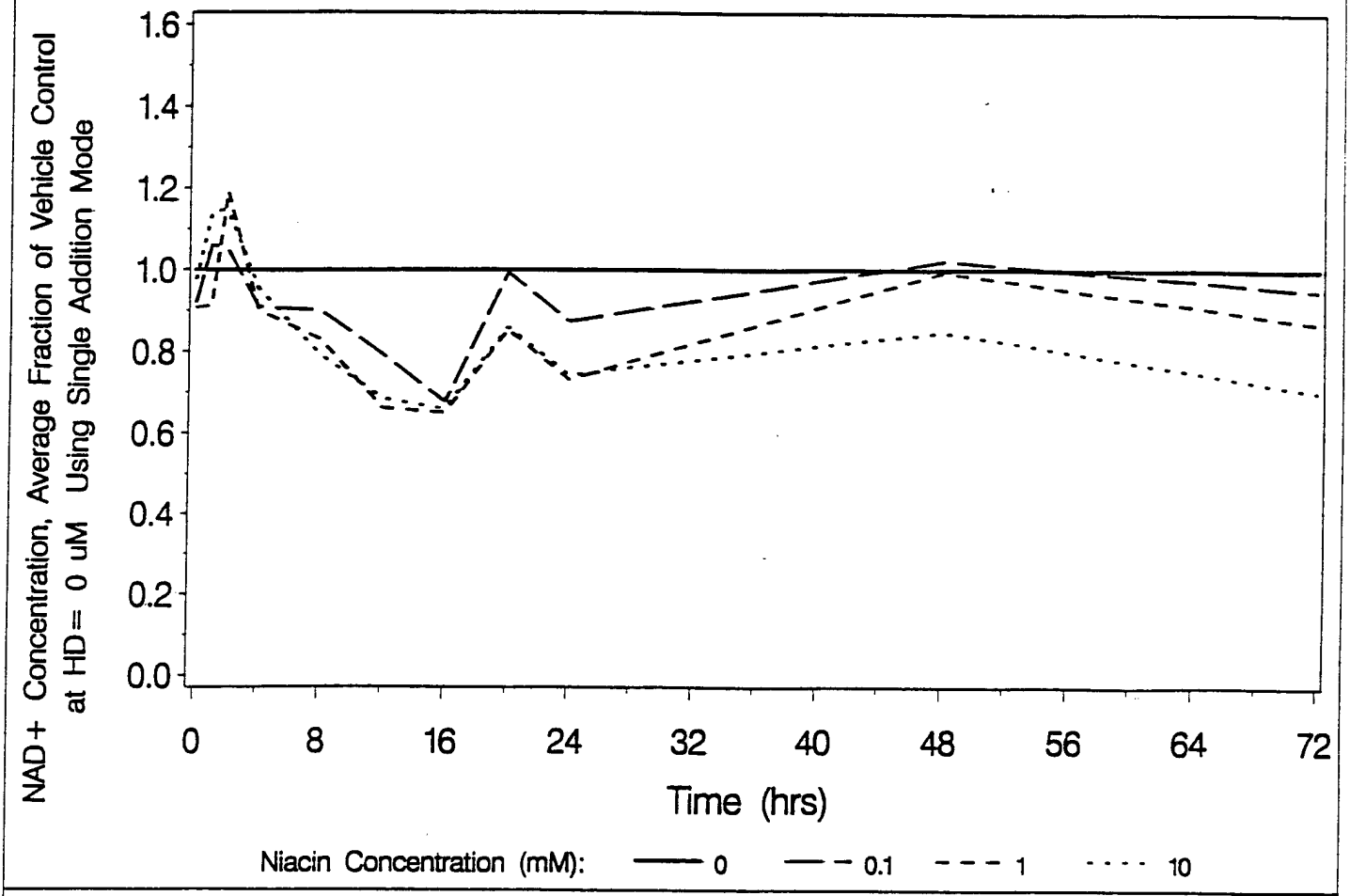


FIGURE F-4.

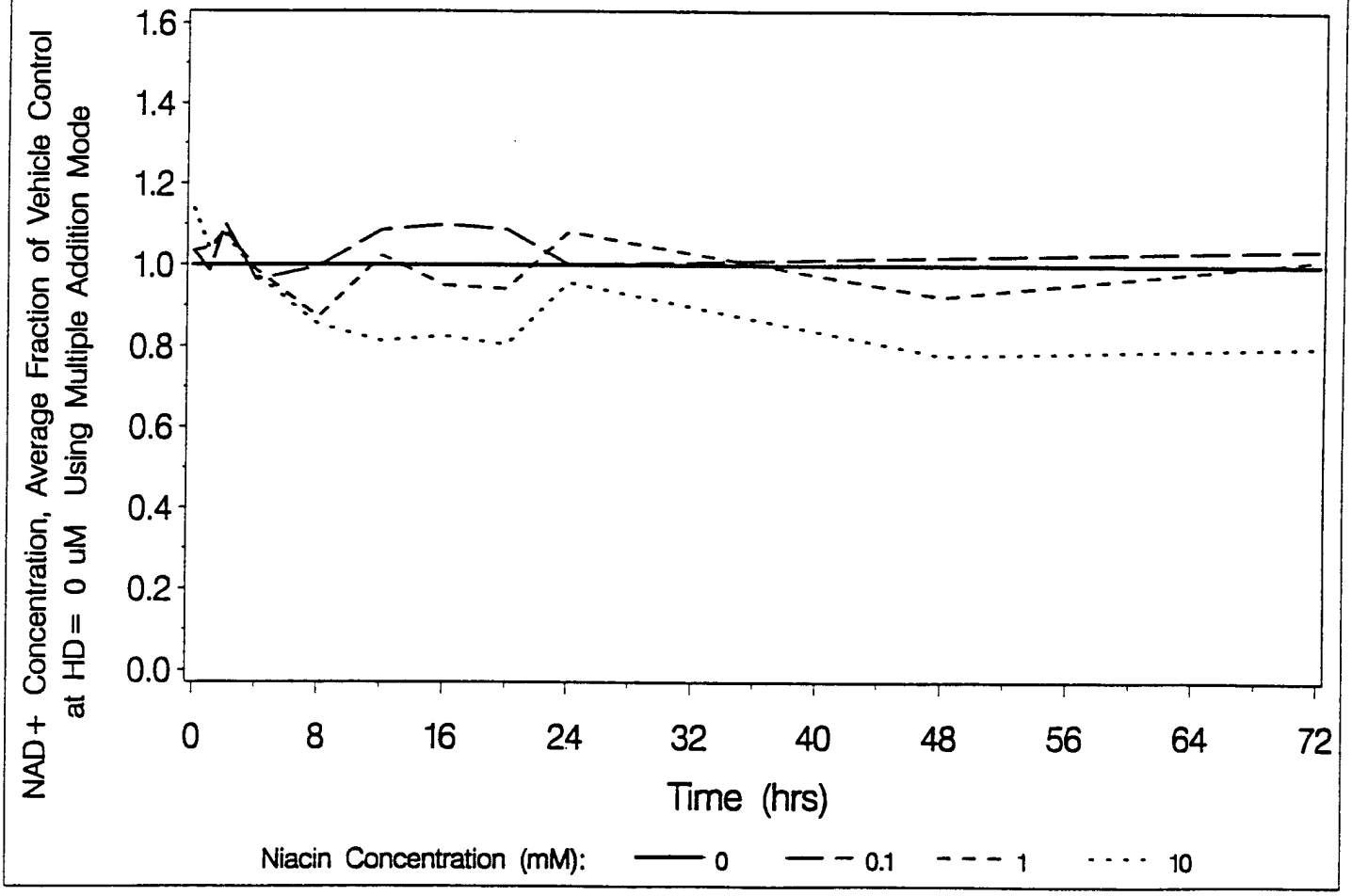


FIGURE F-5.

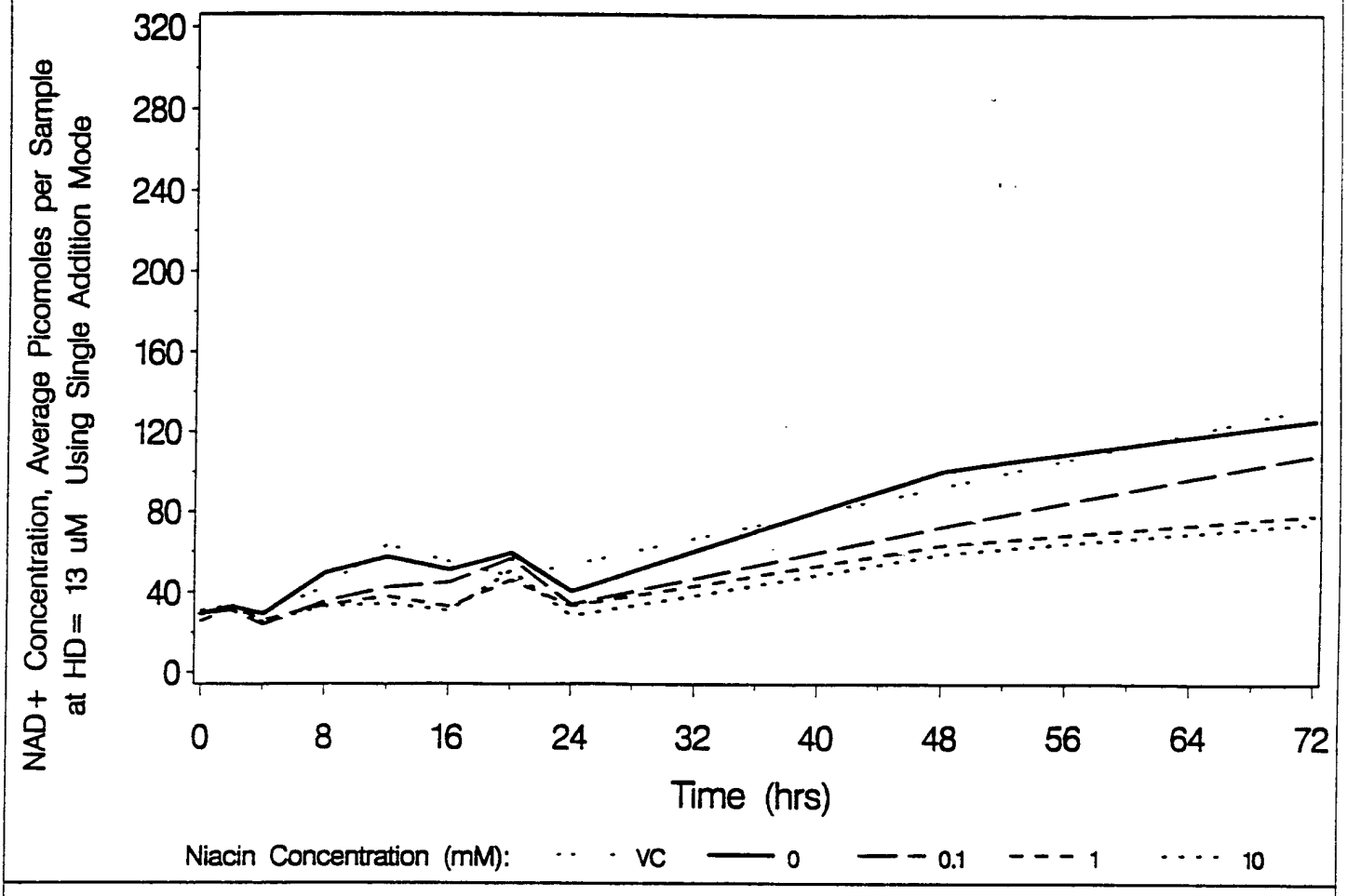


FIGURE F-6.

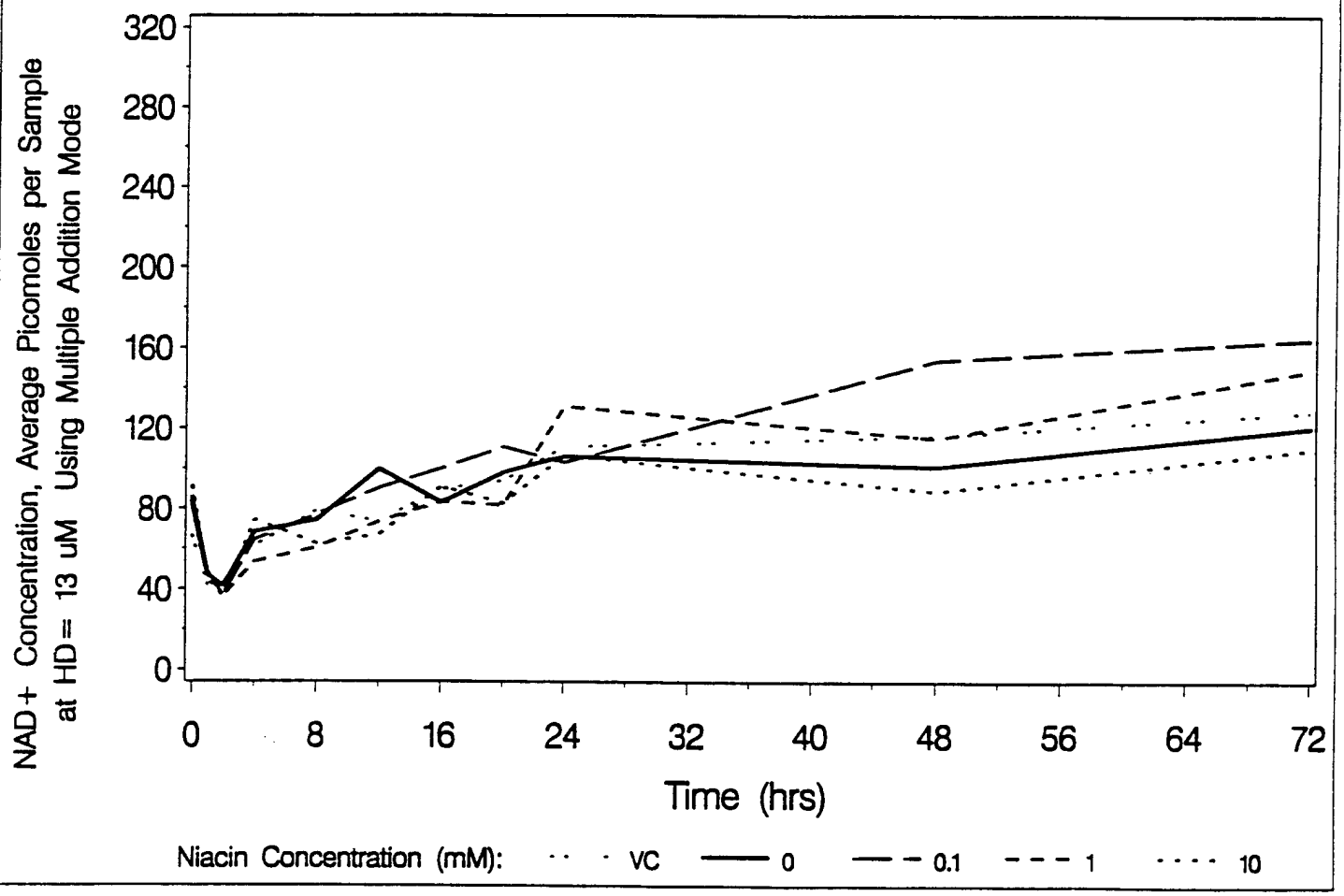


FIGURE F-7.

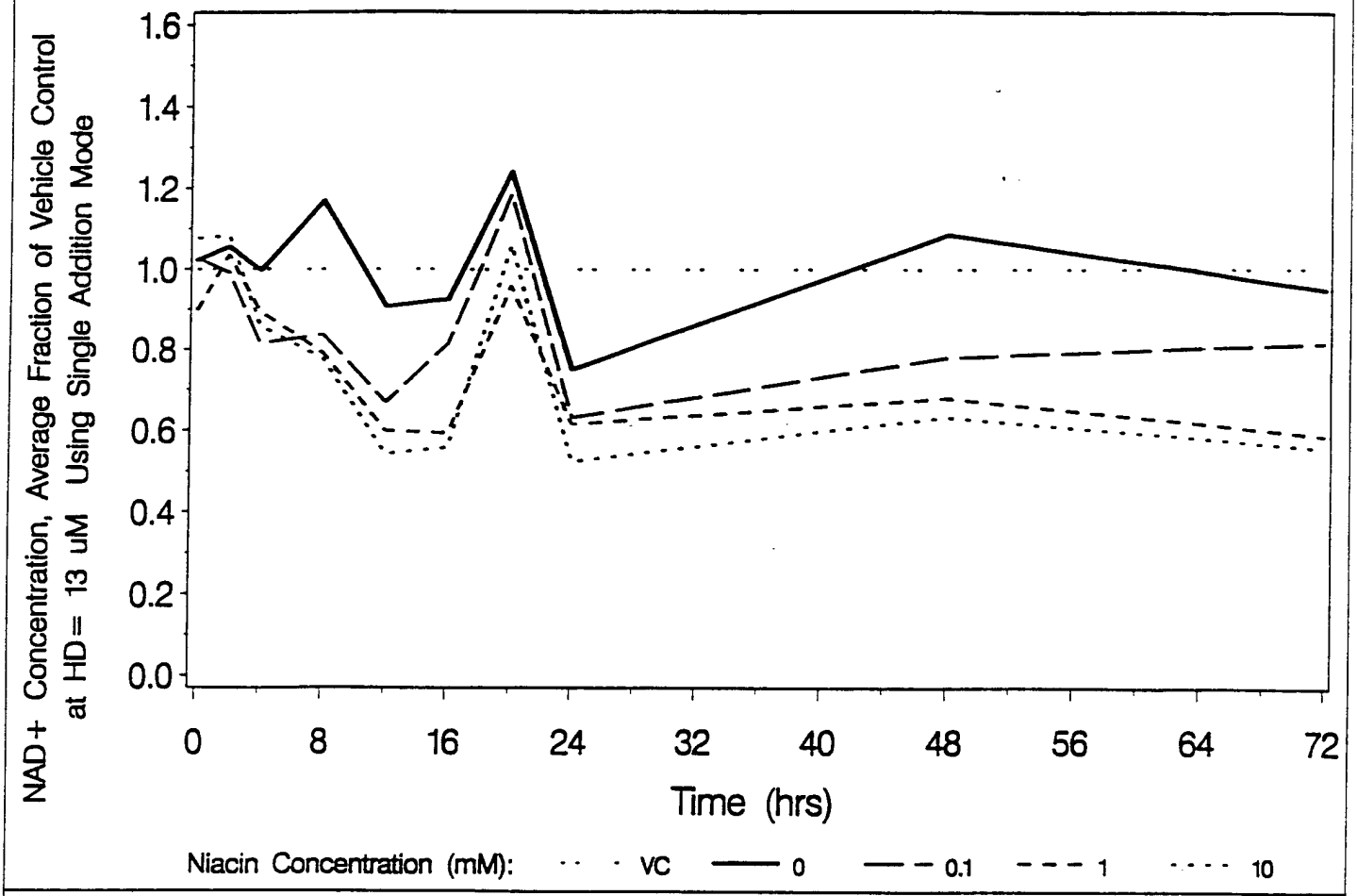


FIGURE F-8.

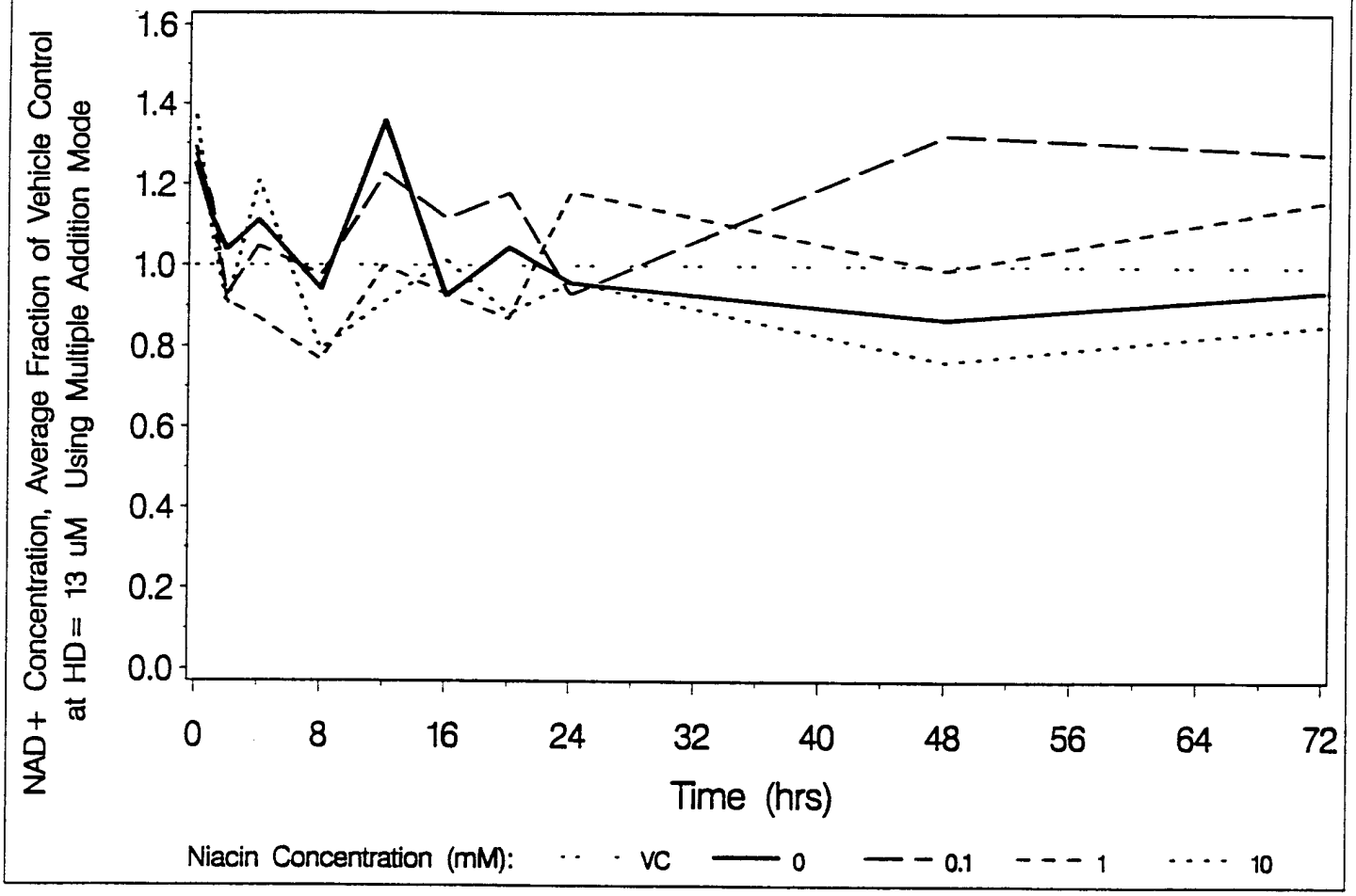


FIGURE F-9.

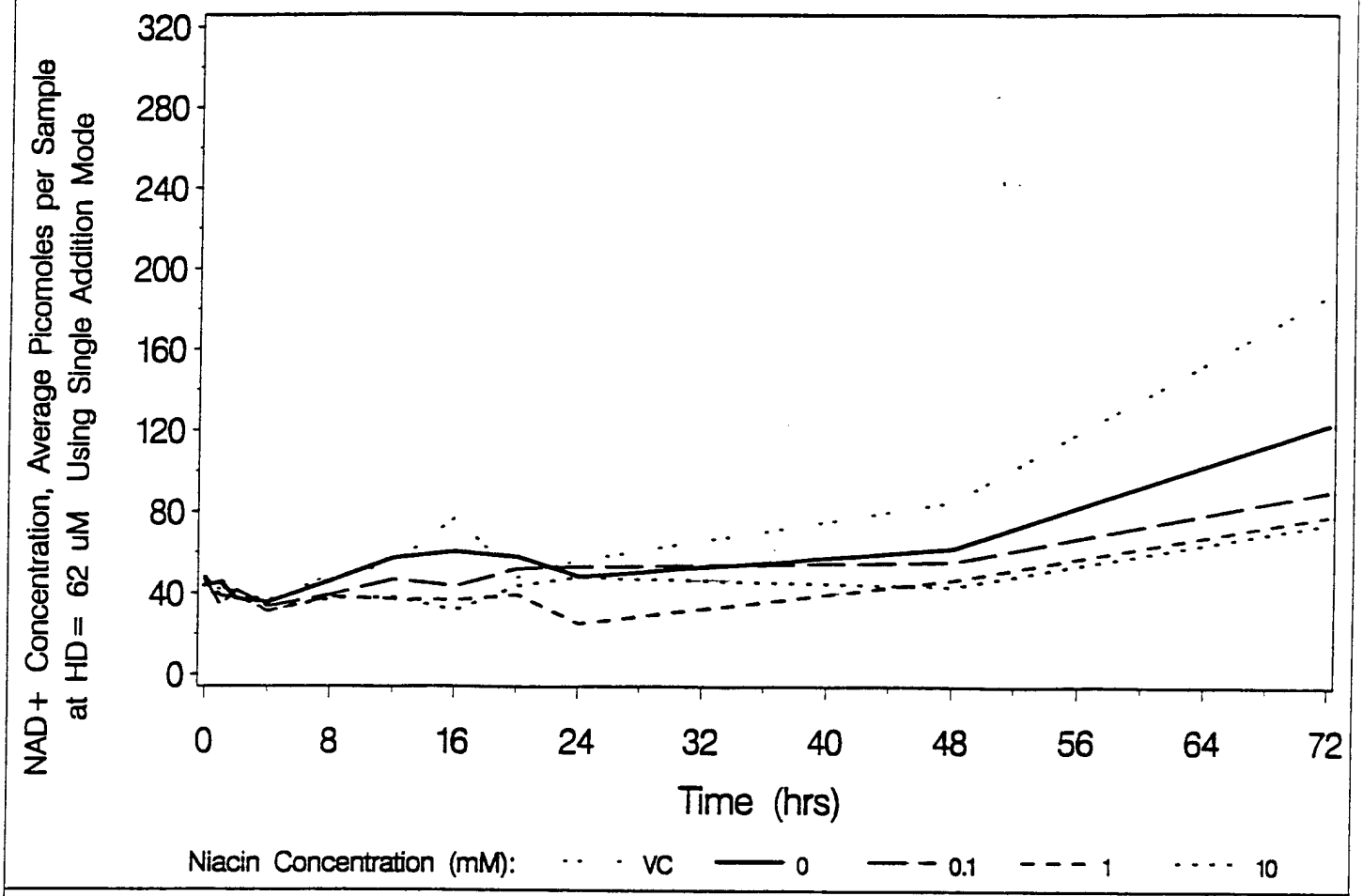


FIGURE F-10.

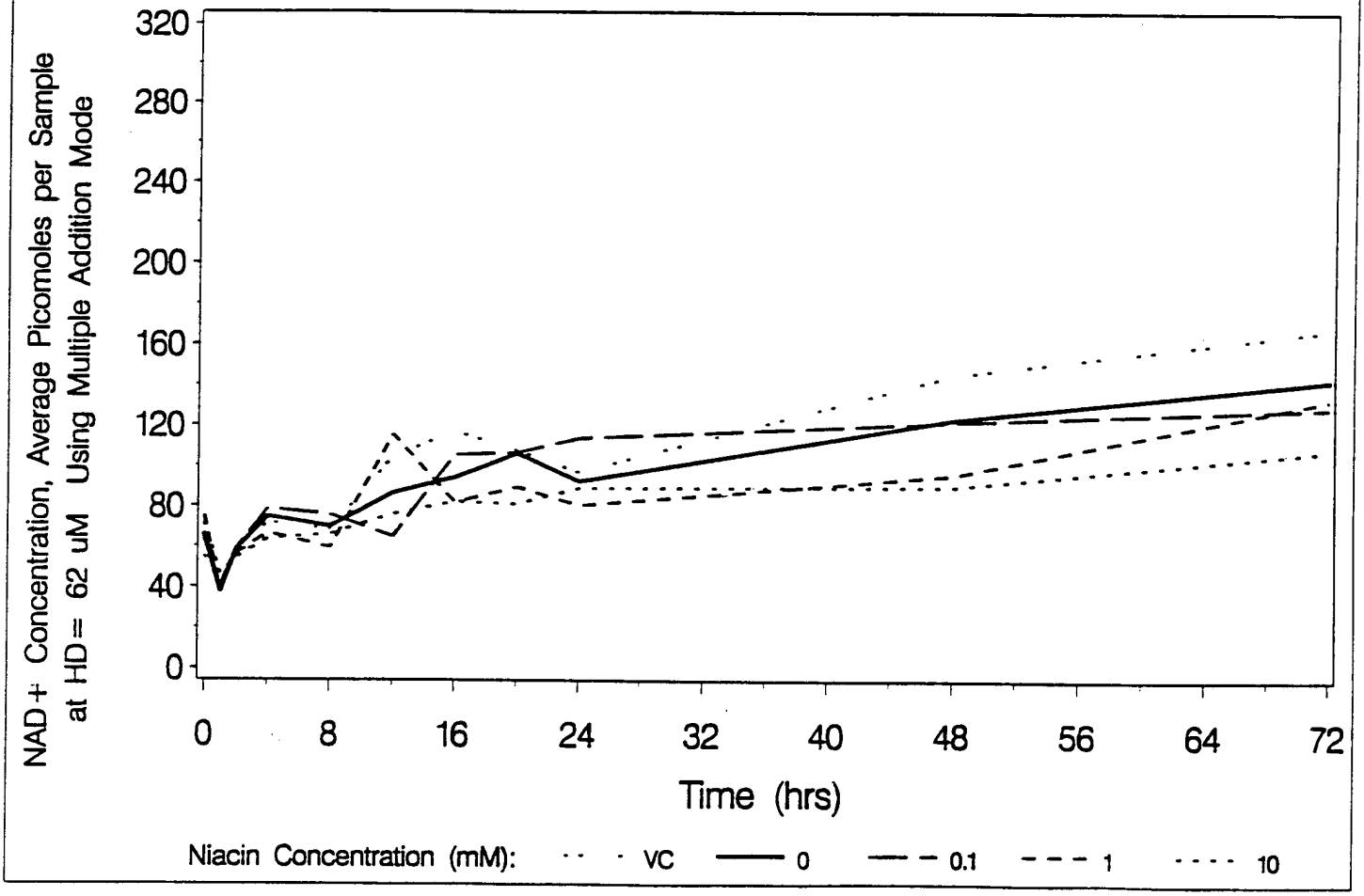


FIGURE F-11.

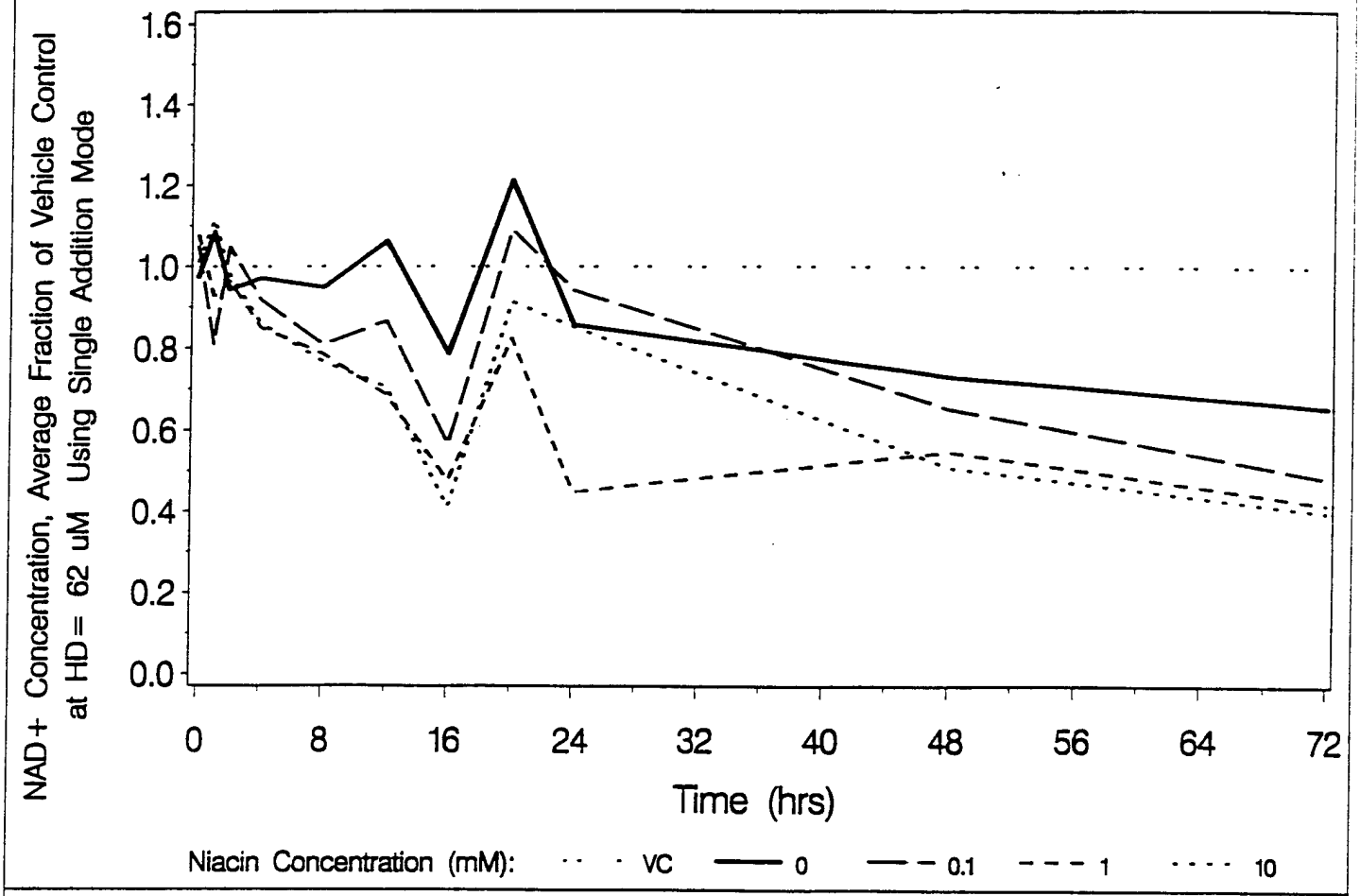


FIGURE F-12.

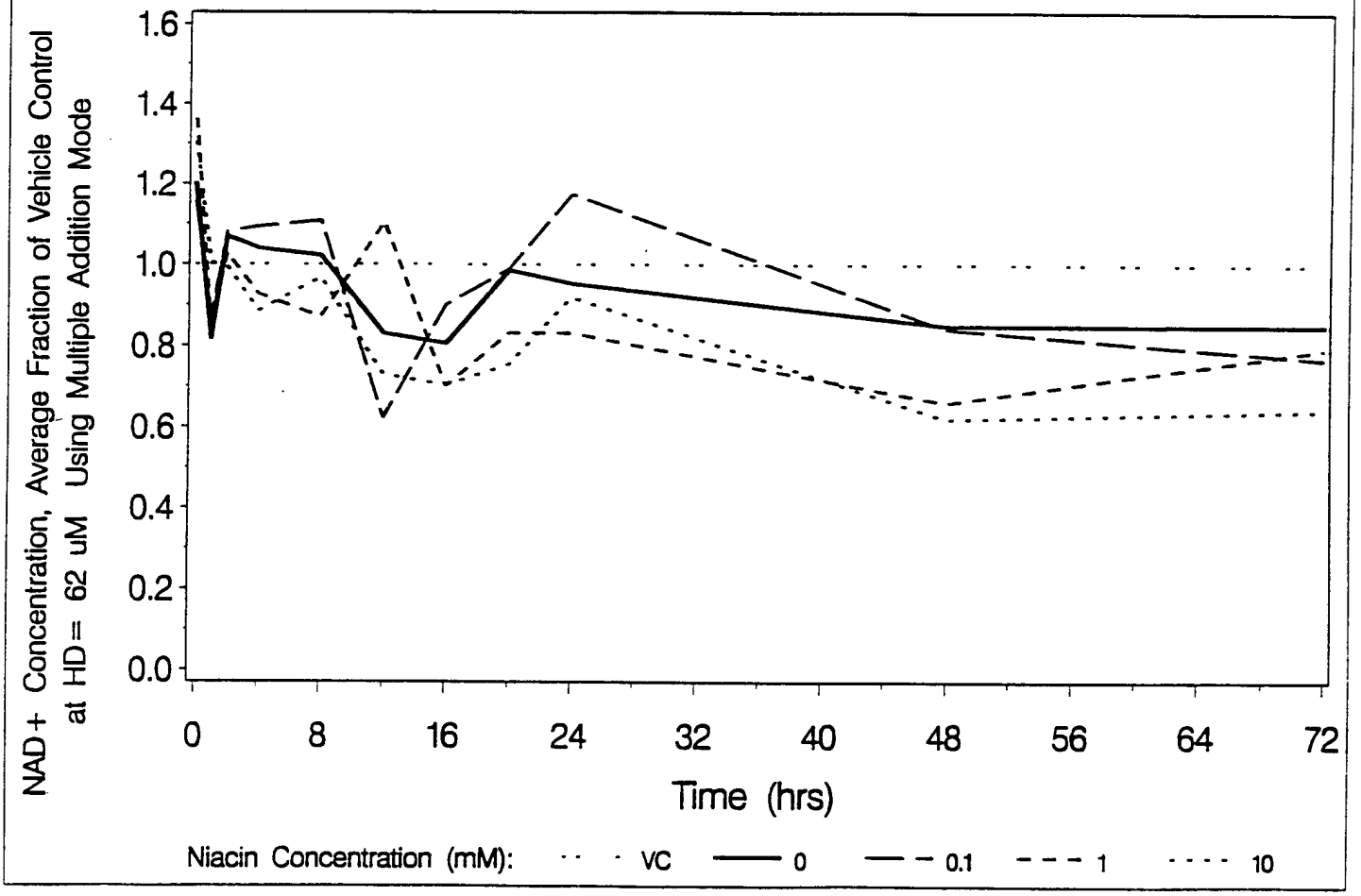


FIGURE F-13.

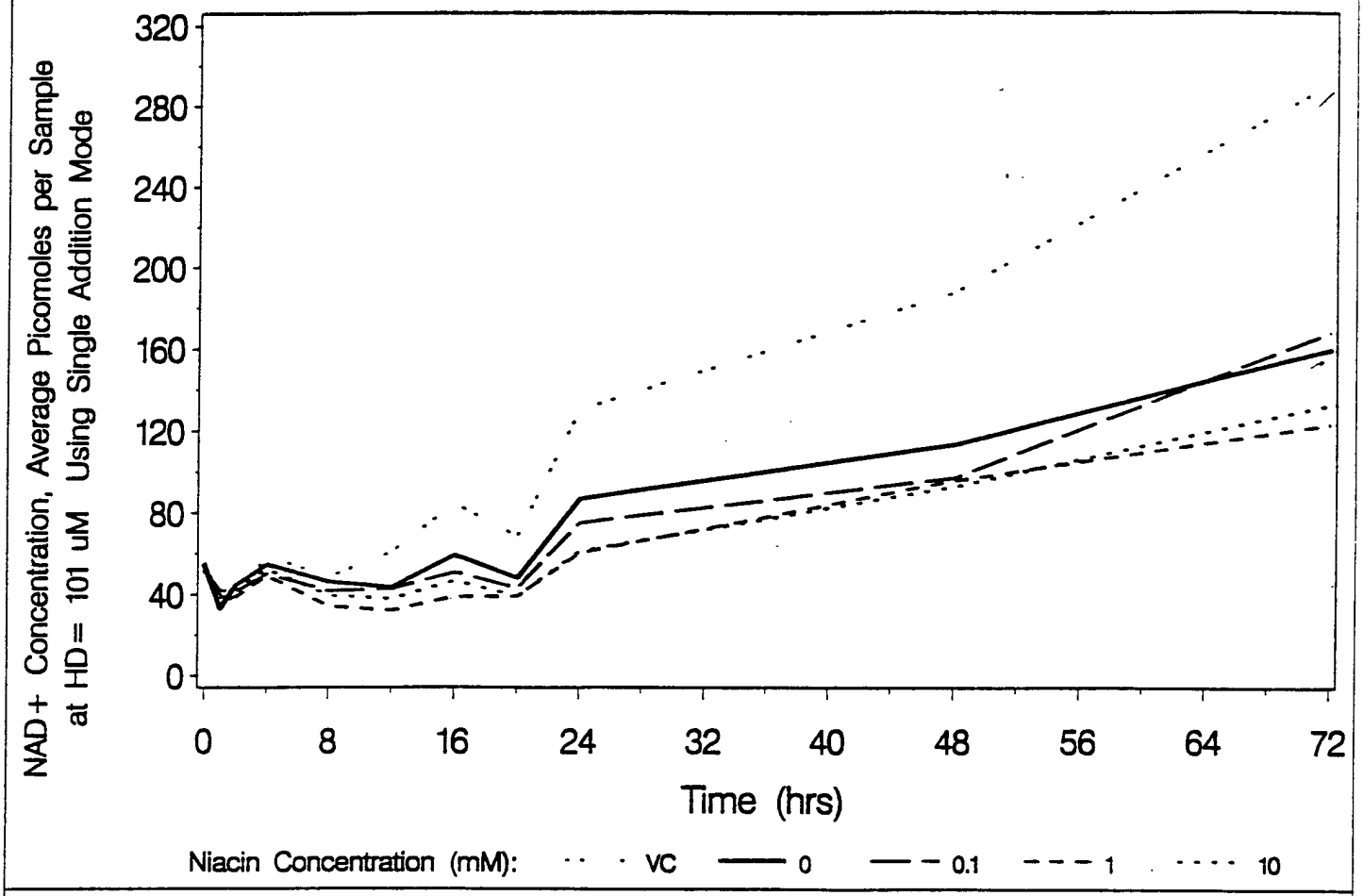


FIGURE F-14.

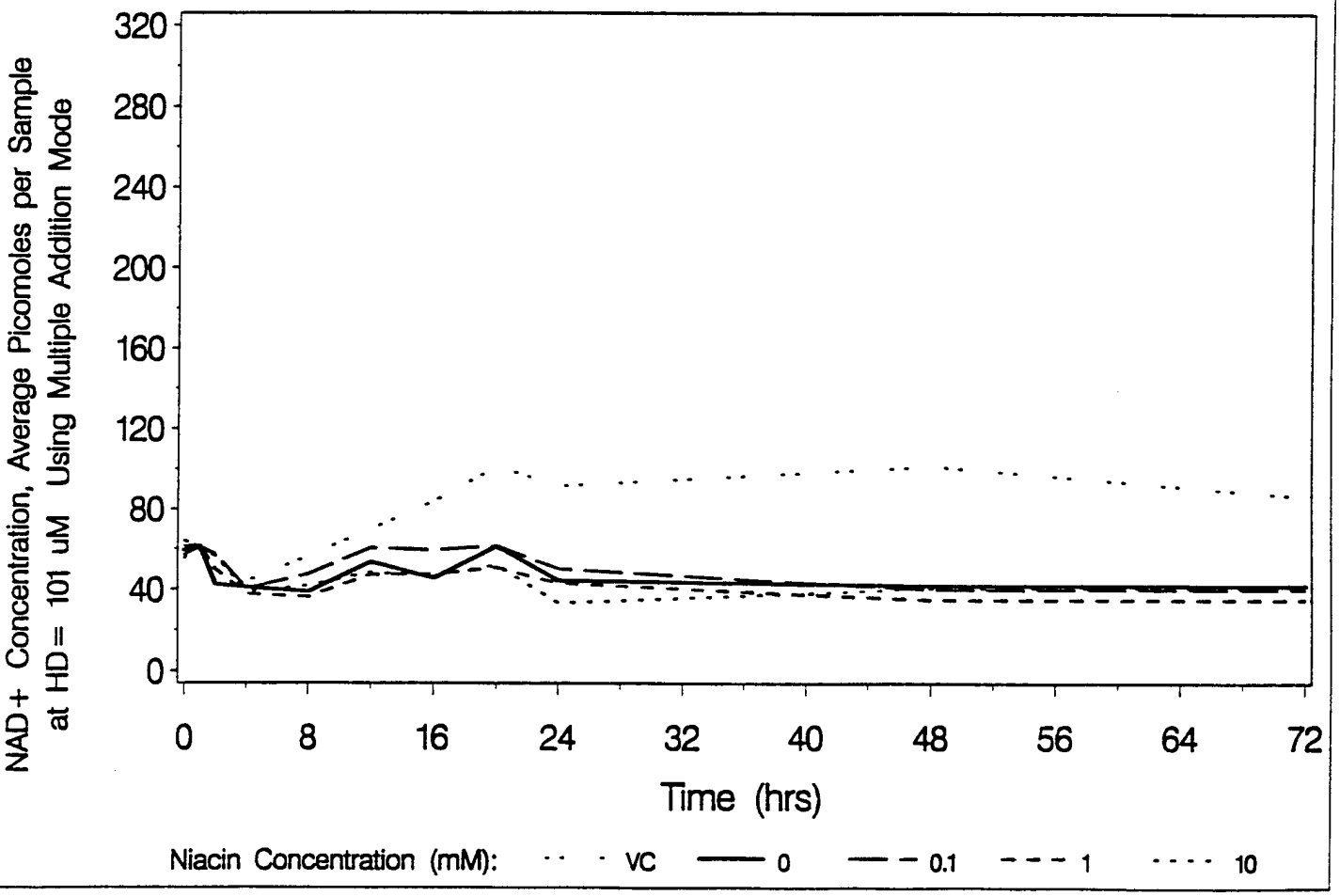


FIGURE F-15.

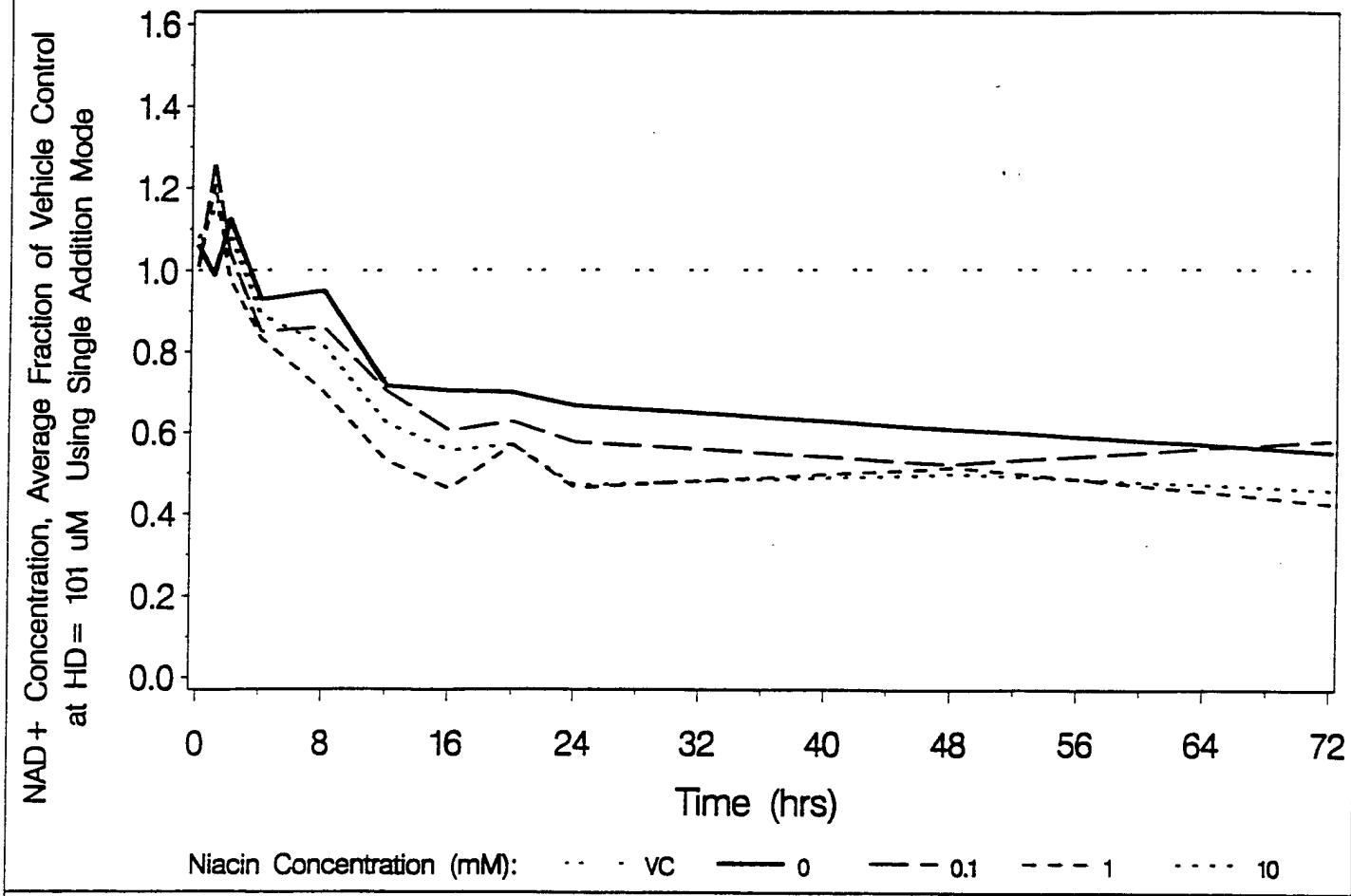


FIGURE F-16.

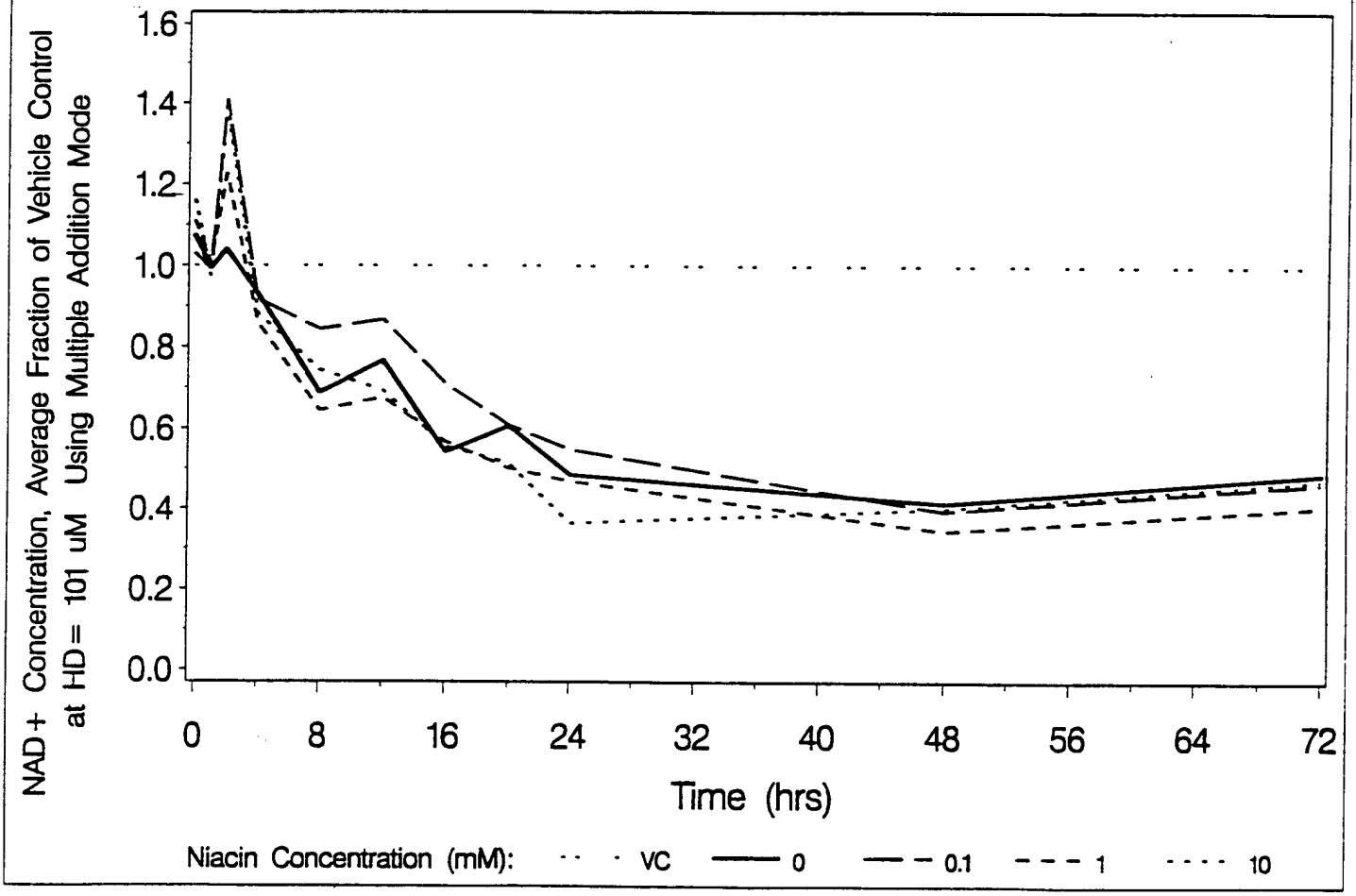


FIGURE F-17.

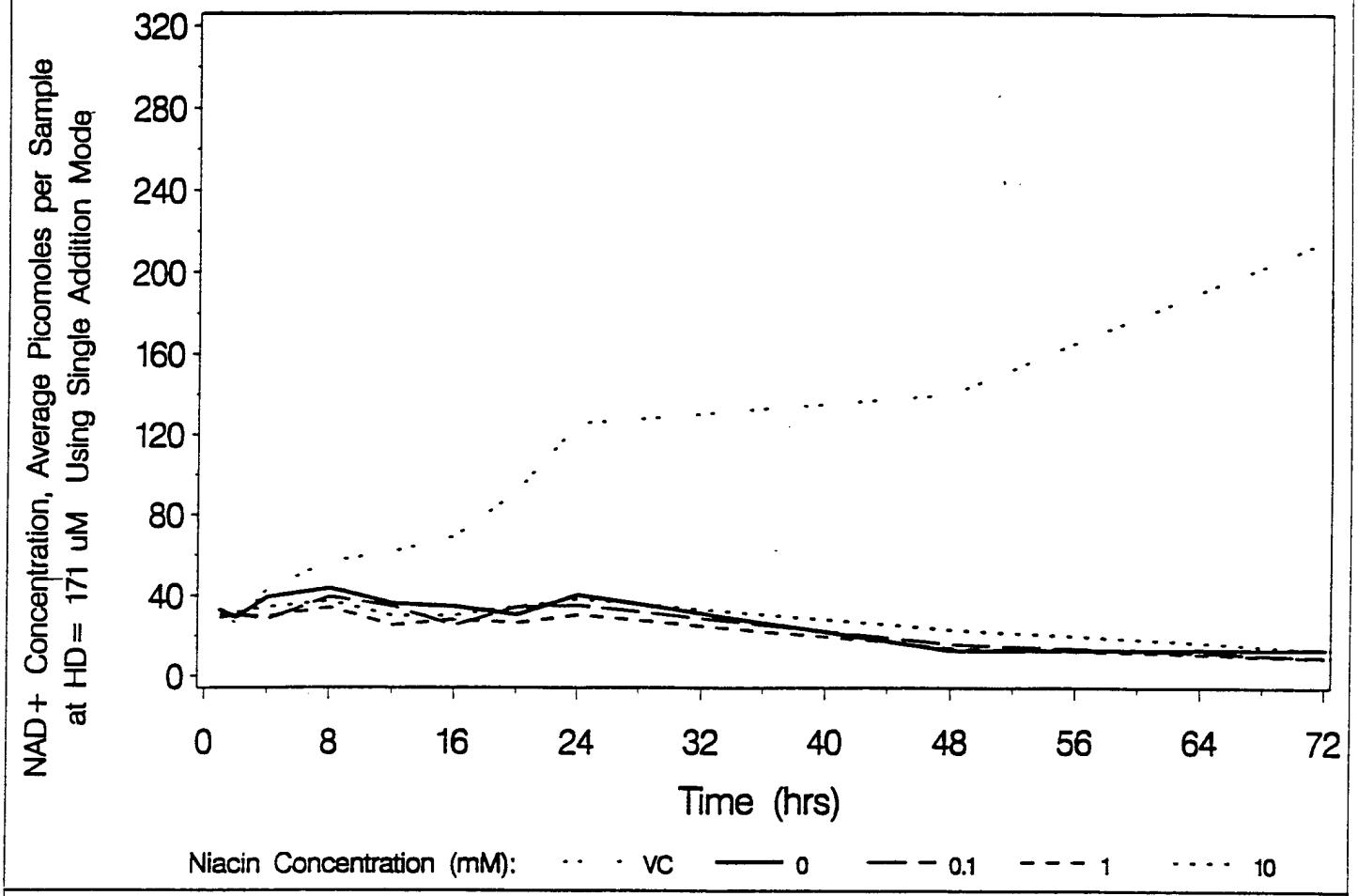


FIGURE F-18.

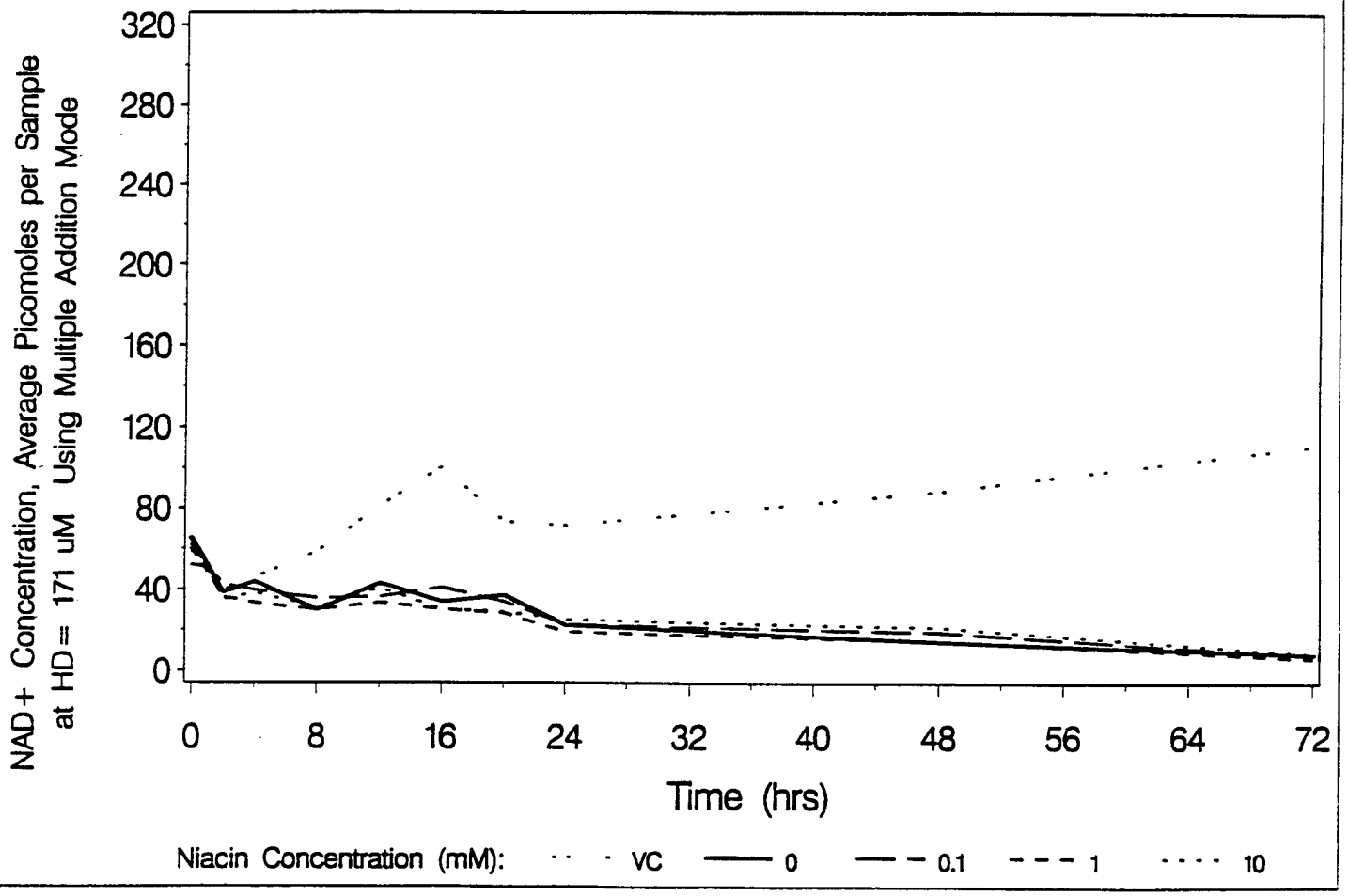


FIGURE F-19.

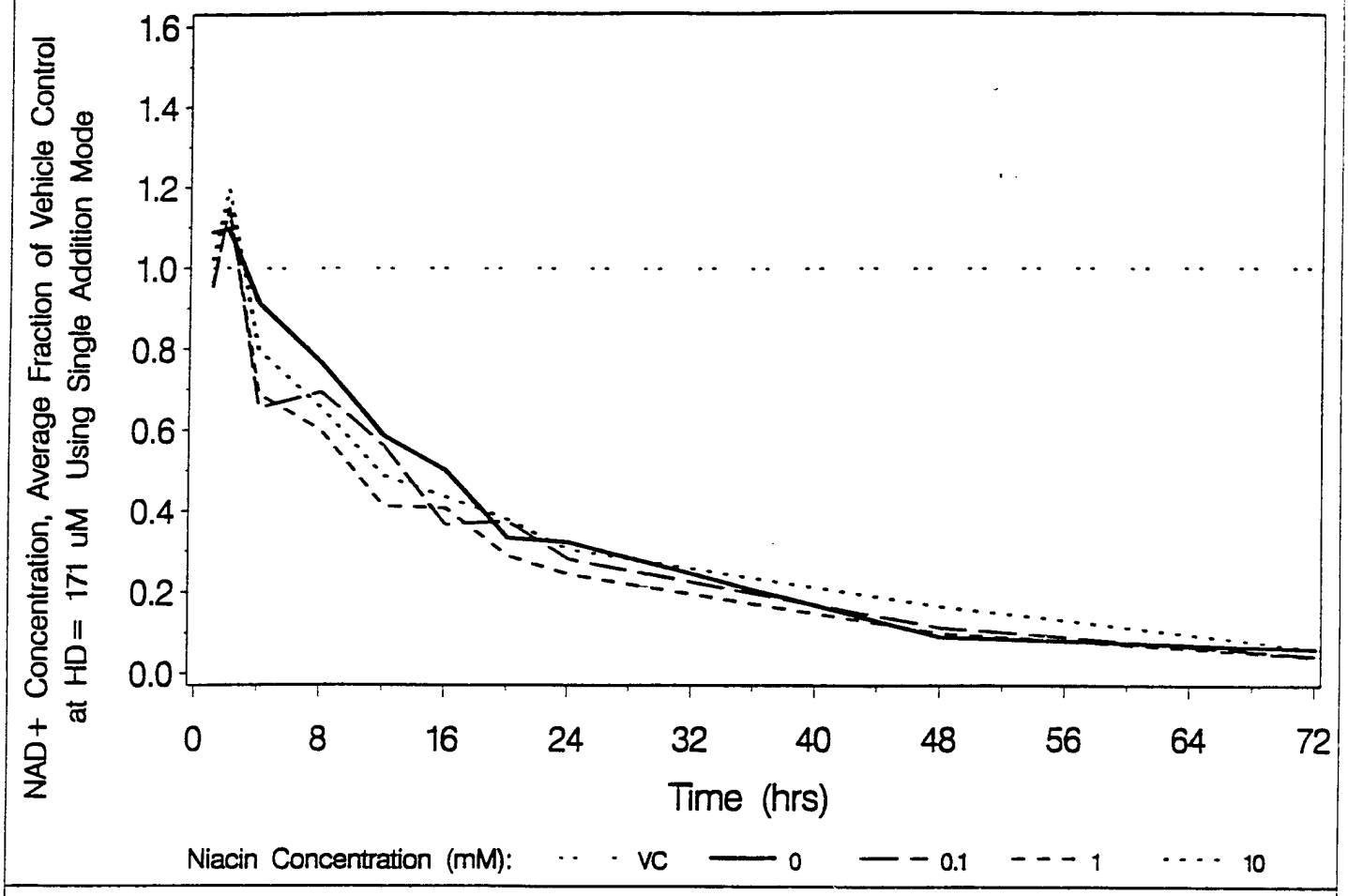
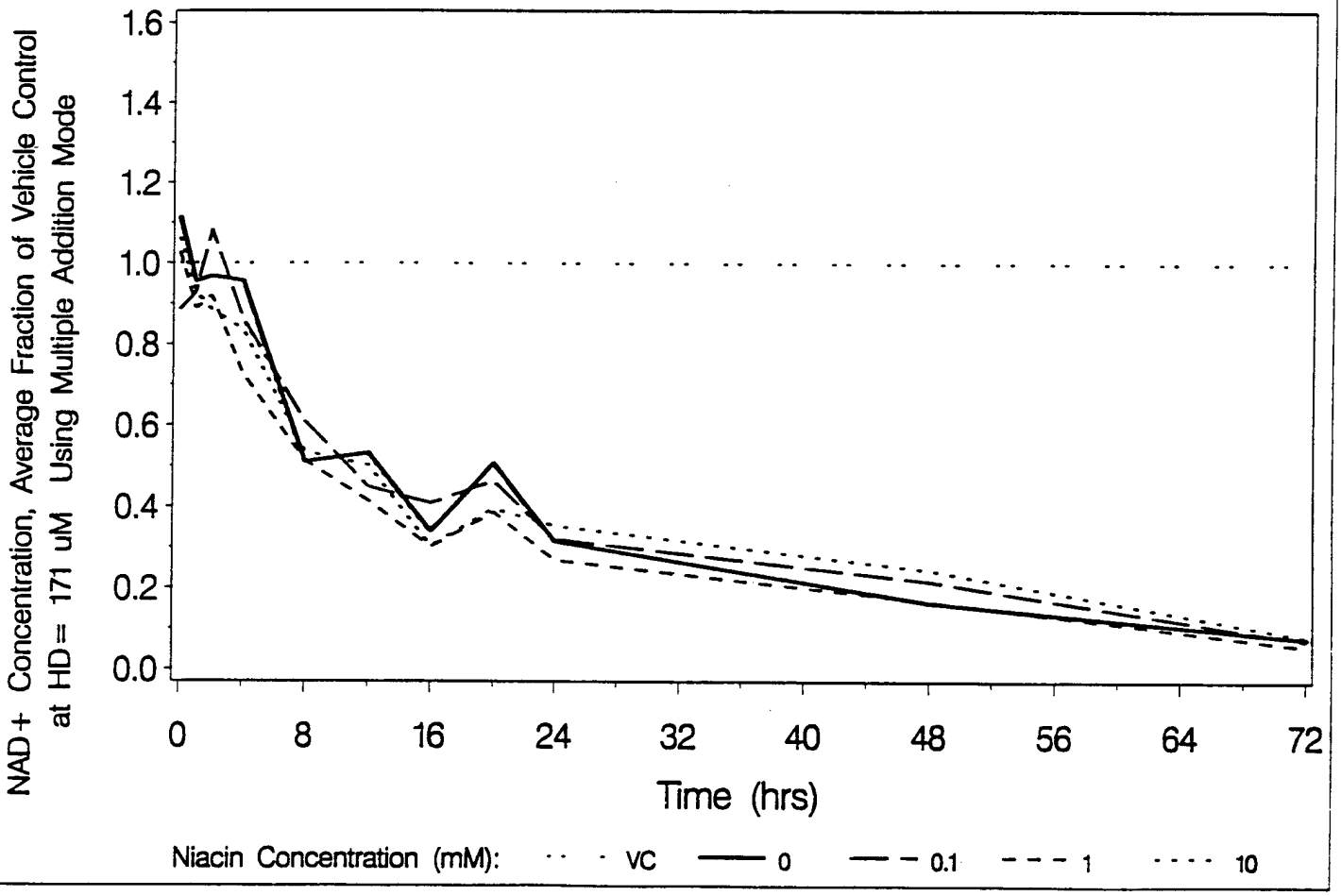


FIGURE F-20.



APPENDIX G

SUMMARY STATISTICS FOR NAD^+ DATA FROM NIACINAMIDE-PRETREATED, HD-EXPOSED CULTURES

TABLE G-1. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	28.5	0.7	2	32.0	1.4	2	32.7	2.1	2	28.3	1.1
	2	3	31.1	5.1	3	33.9	6.2	3	34.9	2.9	3	32.1	4.3
	4	3	28.9	4.4	3	31.4	3.2	3	32.9	1.6	3	34.1	0.4
	8	3	42.2	1.7	3	35.8	0.7	3	41.2	5.6	3	41.4	5.5
	12	3	63.1	5.0	3	51.5	2.5	3	58.7	18.6	3	55.6	9.7
	16	3	54.7	15.0	3	57.4	2.8	3	51.0	2.8	3	49.5	1.8
	20	3	47.5	8.0	3	54.0	2.7	3	59.1	11.6	3	68.1	11.1
	24	3	53.1	14.3	3	52.8	2.4	3	60.4	12.6	3	52.2	3.7
	48	3	91.4	6.3	3	75.4	5.3	3	88.7	13.3	3	105.4	8.7
	72	3	131.4	8.6	3	131.3	10.8	3	127.9	21.7	3	167.8	21.9
13	0	3	29.2	9.1	3	32.3	1.5	3	30.2	5.4	3	32.8	2.0
	2	3	32.9	6.0	3	30.7	2.6	3	37.1	1.8	3	31.9	6.1
	4	3	28.8	3.8	3	27.1	3.6	3	30.2	2.9	3	32.9	2.7
	8	3	49.3	5.0	3	38.8	3.4	3	42.7	6.2	3	43.6	2.3
	12	3	57.3	6.8	3	46.8	9.1	3	51.0	3.4	3	51.1	14.2
	16	3	50.7	3.2	3	46.5	4.2	3	44.6	9.8	3	59.3	8.7
	20	3	59.0	9.0	3	48.8	4.9	3	65.5	9.1	3	71.5	7.9
	24	3	39.9	10.3	2	42.1	7.6	2	54.4	0.6	2	43.3	2.3
	48	2	99.2	16.7	2	60.4	2.8	2	79.5	1.2	2	72.4	1.3
	72	2	124.5	14.7	2	93.2	1.4	2	116.1	0.3	2	77.2	15.7

TABLE G-2. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	1.000	0.025	2	1.122	0.050	2	1.145	0.072	2	0.992	0.040
	2	3	1.000	0.163	3	1.088	0.199	3	1.121	0.094	3	1.031	0.137
	4	3	1.000	0.152	3	1.086	0.110	3	1.137	0.054	3	1.179	0.013
	8	3	1.000	0.040	3	0.850	0.017	3	0.976	0.134	3	0.983	0.130
	12	3	1.000	0.080	3	0.816	0.039	3	0.929	0.295	3	0.881	0.154
	16	3	1.000	0.275	3	1.050	0.051	3	0.932	0.051	3	0.905	0.033
	20	3	1.000	0.167	3	1.137	0.056	3	1.243	0.245	3	1.434	0.233
	24	3	1.000	0.270	3	0.995	0.044	3	1.138	0.238	3	0.984	0.069
	48	3	1.000	0.069	3	0.825	0.057	3	0.971	0.146	3	1.154	0.096
	72	3	1.000	0.066	3	0.999	0.082	3	0.974	0.165	3	1.277	0.166
13	0	3	1.023	0.319	3	1.133	0.051	3	1.057	0.191	3	1.150	0.069
	2	3	1.056	0.192	3	0.987	0.084	3	1.191	0.059	3	1.027	0.195
	4	3	0.997	0.132	3	0.935	0.126	3	1.044	0.101	3	1.137	0.095
	8	3	1.169	0.118	3	0.921[b]	0.081	3	1.012	0.147	3	1.035	0.055
	12	3	0.907	0.107	3	0.741	0.144	3	0.807	0.054	3	0.810	0.225
	16	3	0.926	0.058	3	0.850	0.076	3	0.816	0.179	3	1.084	0.159
	20	3	1.241	0.189	3	1.028	0.103	3	1.378	0.192	3	1.504	0.166
	24	3	0.751	0.194	2	0.794	0.144	2	1.026[b]	0.012	2	0.816	0.043
	48	2	1.085	0.182	2	0.661[b]	0.030	2	0.870	0.014	2	0.792[b]	0.015
	72	2	0.948	0.112	2	0.710	0.011	2	0.884	0.002	2	0.588[b]	0.120

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-3. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	66.5	21.6	3	72.8	2.6	3	73.9	4.6	3	77.2	2.5
	1	3	41.8	5.5	3	42.8	0.5	3	41.1	8.7	3	42.0	6.9
	2	3	39.8	8.2	3	43.6	1.8	3	44.3	1.3	3	38.3	3.2
	4	3	61.4	2.5	3	58.0	17.8	3	59.5	5.3	3	50.9	15.0
	8	3	78.9	15.9	3	80.4	8.2	3	94.3	4.7	3	93.7	10.0
	12	3	73.5	23.5	3	97.1	14.5	3	95.8	12.9	3	82.5	8.5
	16	3	89.6	16.8	3	90.0	21.0	3	95.7	7.5	3	92.3	2.3
	20	3	93.9	11.3	3	88.5	4.8	3	92.6	10.6	3	95.0	4.1
	24	3	110.8	17.9	3	141.6	14.7	3	136.3	4.2	3	153.3	4.6
	48	3	116.5	9.2	3	128.2	1.8	3	137.0	17.3	3	141.2	10.1
	72	3	129.0	6.1	3	134.6	8.8	3	142.1	17.5	3	167.5	6.1
13	0	3	83.2	6.3	3	80.8	5.2	3	85.3	2.6	3	90.0	5.2
	1	3	46.7	2.2	3	36.7	4.2	3	48.5	5.6	3	39.2	10.5
	2	3	41.3	7.9	3	34.6	8.3	2	46.1	8.7	3	39.2	3.4
	4	3	68.2	8.0	3	74.9	10.5	3	68.9	3.3	3	69.6	14.7
	8	3	74.2	15.2	3	54.7	8.6	3	67.3	11.6	3	86.4	11.1
	12	3	99.7	21.1	3	87.4	12.7	3	108.7	18.4	3	105.5	10.2
	16	3	82.9	13.6	3	68.7	5.3	2	84.0	0.4	3	110.2	4.8
	20	3	98.1	25.4	3	102.0	18.7	3	93.5	21.0	3	104.0	12.4
	24	3	106.0	5.0	3	108.6	16.0	3	127.0	14.8	3	143.5	9.5
	48	3	101.1	8.0	3	124.0	19.1	3	142.6	18.2	3	166.0	14.6
	72	3	121.1	29.0	3	119.4	4.3	3	170.2	11.2	3	173.6	23.4

TABLE G-4. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.325	3	1.095	0.039	3	1.112	0.070	3	1.161	0.037
	1	3	1.000	0.131	3	1.025	0.012	3	0.985	0.208	3	1.006	0.164
	2	3	1.000	0.205	3	1.095	0.046	3	1.112	0.032	3	0.962	0.079
	4	3	1.000	0.040	3	0.944	0.290	3	0.968	0.087	3	0.830	0.244
	8	3	1.000	0.201	3	1.019	0.104	3	1.196	0.059	3	1.188	0.126
	12	3	1.000	0.320	3	1.321	0.198	3	1.303	0.176	3	1.123	0.115
	16	3	1.000	0.187	3	1.004	0.234	3	1.067	0.084	3	1.029	0.026
	20	3	1.000	0.120	3	0.942	0.052	3	0.987	0.113	3	1.012	0.044
	24	3	1.000	0.161	3	1.278	0.133	3	1.230	0.038	3	1.383	0.042
	48	3	1.000	0.079	3	1.100	0.015	3	1.176	0.149	3	1.212	0.087
	72	3	1.000	0.047	3	1.044	0.068	3	1.102	0.136	3	1.299	0.047
13	0	3	1.251	0.094	3	1.215	0.078	3	1.282	0.039	3	1.352	0.078
	1	3	1.119	0.051	3	0.878	0.101	3	1.161	0.134	3	0.939	0.252
	2	3	1.038	0.199	3	0.868	0.208	2	1.157	0.218	3	0.984	0.085
	4	3	1.111	0.130	3	1.220	0.170	3	1.122	0.053	3	1.134	0.239
	8	3	0.940	0.192	3	0.693[b]	0.110	3	0.853	0.146	3	1.096	0.140
	12	3	1.356[a]	0.287	3	1.189	0.173	3	1.479	0.250	3	1.435	0.139
	16	3	0.925	0.152	3	0.767	0.060	2	0.937	0.005	3	1.229[b]	0.054
	20	3	1.045	0.270	3	1.087	0.199	3	0.996	0.224	3	1.108	0.132
	24	3	0.957	0.045	3	0.980	0.145	3	1.146	0.134	3	1.295[b]	0.085
	48	3	0.868	0.069	3	1.064	0.164	3	1.224[b]	0.156	3	1.425[b]	0.125
	72	3	0.939	0.225	3	0.925	0.034	3	1.320[b]	0.087	3	1.346[b]	0.181

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-5. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			N = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	1	3	41.7	8.0	3	33.2	3.9	3	37.5	3.1	3	29.9	2.0
	2	3	39.4	11.9	3	38.9	2.9	3	42.5	0.4	3	43.3	2.9
	4	3	36.4	4.2	3	34.2	2.3	3	39.9	2.5	3	40.6	0.1
	8	3	48.4	5.2	3	41.1	3.2	3	46.0	2.0	3	50.6	6.0
	12	3	53.8	2.8	3	49.5	8.1	3	59.4	9.4	3	57.9	6.9
	16	3	76.7	10.5	3	61.6	4.0	3	63.5	1.1	3	57.4	9.6
	20	3	47.4	6.8	3	43.2	12.7	3	55.2	7.5	3	67.6	6.1
	24	3	55.8	8.9	3	65.2	6.5	3	61.5	4.4	3	63.0	8.7
	48	2	85.0	1.9	3	80.4	8.6	3	83.3	22.1	3	95.9	8.6
	72	3	187.6	19.5	3	141.1	29.3	3	160.0	26.6	2	186.1	22.5
62	0	1	43.6	-	2	43.5	4.6	2	49.6	7.2	2	47.2	2.5
	1	3	45.3	6.7	3	39.2	2.3	3	33.9	8.4	3	31.9	1.9
	2	3	37.1	10.8	3	34.9	0.7	3	40.6	2.0	3	42.7	4.3
	4	3	35.4	5.5	3	32.0	2.5	3	33.7	4.9	3	41.8	1.2
	8	3	45.9	3.7	3	34.7	0.5	3	40.7	7.2	3	48.7	7.8
	12	3	57.2	2.7	3	46.1	2.1	3	61.5	4.0	3	52.9	6.2
	16	3	60.2	3.6	3	50.4	7.0	3	54.9	7.1	3	59.2	7.5
	20	3	57.5	13.0	3	51.9	1.4	3	43.7	11.8	3	54.8	17.4
	24	3	47.8	9.7	3	42.3	6.1	3	51.3	6.1	3	59.1	13.7
	48	2	62.0	10.9	3	61.6	9.4	3	58.6	19.9	2	72.8	5.8
	72	3	123.3	8.6	3	88.5	5.0	2	85.4	9.9	3	119.2	9.5

TABLE G-6. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.087	3	0.971	0.036	3	1.063	0.049	3	1.063	0.082
	1	3	1.000	0.192	3	0.797	0.095	3	0.900	0.074	3	0.716	0.047
	2	3	1.000	0.303	3	0.988	0.074	3	1.081	0.009	3	1.100	0.074
	4	3	1.000	0.115	3	0.939	0.062	3	1.097	0.069	3	1.116	0.004
	8	3	1.000	0.107	3	0.850	0.066	3	0.950	0.042	3	1.046	0.123
	12	3	1.000	0.052	3	0.920	0.150	3	1.104	0.174	3	1.077	0.128
	16	3	1.000	0.137	3	0.803	0.052	3	0.828	0.014	3	0.748	0.125
	20	3	1.000	0.144	3	0.912	0.268	3	1.166	0.159	3	1.427	0.129
	24	3	1.000	0.160	3	1.167	0.117	3	1.101	0.079	3	1.128	0.156
	48	2	1.000	0.022	3	0.946	0.101	3	0.980	0.260	3	1.128	0.101
	72	3	1.000	0.104	3	0.752	0.156	3	0.853	0.142	2	0.992	0.120
62	0	1	0.974	-	2	0.972	0.103	2	1.107	0.161	2	1.054	0.055
	1	3	1.086	0.160	3	0.941	0.054	3	0.815	0.203	3	0.765	0.046
	2	3	0.943	0.274	3	0.888	0.017	3	1.031	0.050	3	1.086	0.110
	4	3	0.972	0.150	3	0.879	0.069	3	0.926	0.135	3	1.149[b]	0.032
	8	3	0.948	0.076	3	0.717	0.010	3	0.842	0.149	3	1.008	0.161
	12	3	1.063	0.051	3	0.857[b]	0.040	3	1.144	0.075	3	0.984	0.115
	16	3	0.784	0.047	3	0.657	0.091	3	0.715	0.092	3	0.772	0.097
	20	3	1.213	0.274	3	1.095	0.029	3	0.921	0.248	3	1.156	0.367
	24	3	0.857	0.173	3	0.758	0.109	3	0.918	0.108	3	1.057	0.245
	48	2	0.730	0.128	3	0.724	0.111	3	0.689	0.234	2	0.856	0.068
	72	3	0.658[a]	0.046	3	0.472[b]	0.027	2	0.456[b]	0.053	3	0.636	0.051

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-7. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	54.9	8.0	3	57.4	4.0	3	57.1	3.0	3	56.7	5.2
	1	3	46.0	3.3	3	41.6	7.1	3	51.7	5.0	3	50.2	3.5
	2	3	54.5	2.7	3	60.4	4.4	3	60.2	1.6	3	60.8	4.0
	4	3	71.9	7.9	3	78.5	2.8	3	80.8	0.3	3	82.5	2.6
	8	3	68.2	10.0	3	78.6	12.0	3	82.3	7.5	3	78.7	17.6
	12	3	104.2	19.5	3	93.1	2.0	3	114.6	19.4	3	120.0	28.8
	16	3	116.7	7.1	3	124.9	2.9	3	126.0	9.6	3	125.7	13.3
	20	3	107.5	26.1	3	115.4	24.3	3	125.9	6.2	3	133.7	21.0
	24	3	96.7	42.4	3	141.3	5.9	3	141.3	6.9	1	114.3	-
	48	3	145.0	20.8	3	150.6	12.4	3	144.7	7.0	3	170.1	11.8
	72	3	167.7	5.6	3	168.8	10.4	3	170.8	13.5	3	204.5	14.0
62	0	3	65.8	8.5	3	69.8	7.1	3	68.3	4.6	3	72.0	7.7
	1	3	37.5	14.4	3	31.9	4.7	3	35.8	8.9	3	45.1	18.3
	2	3	58.3	5.3	3	46.4	10.2	2	58.3	3.7	3	61.2	6.1
	4	3	74.7	5.0	3	67.5	3.3	3	77.9	4.3	3	83.0	5.9
	8	3	69.7	12.8	3	69.8	15.3	3	66.4	24.8	3	90.7	11.6
	12	3	86.4	1.6	3	84.3	13.9	3	106.0	10.5	3	103.0	19.1
	16	3	93.9	14.6	3	98.3	5.8	3	100.9	2.7	3	122.0	4.2
	20	3	106.0	32.1	3	110.8	13.2	3	104.9	14.5	3	124.5	5.9
	24	3	92.1	17.3	3	92.9	11.6	3	91.8	17.9	3	129.0	11.2
	48	3	123.1	28.4	3	111.3	23.0	3	98.4	19.9	3	135.5	21.6
	72	3	142.5	24.8	3	143.3	12.8	3	148.2	27.0	3	167.1	18.9

TABLE G-8. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.146	3	1.046	0.073	3	1.040	0.055	3	1.033	0.095
	1	3	1.000	0.071	3	0.905	0.154	3	1.124	0.110	3	1.092	0.075
	2	3	1.000	0.049	3	1.108	0.081	3	1.104	0.029	3	1.115	0.074
	4	3	1.000	0.109	3	1.093	0.039	3	1.125	0.005	3	1.148	0.037
	8	3	1.000	0.146	3	1.153	0.176	3	1.206	0.111	3	1.154	0.258
	12	3	1.000	0.187	3	0.894	0.019	3	1.101	0.186	3	1.152	0.276
	16	3	1.000	0.061	3	1.070	0.025	3	1.079	0.082	3	1.077	0.114
	20	3	1.000	0.243	3	1.074	0.226	3	1.171	0.058	3	1.244	0.195
	24	3	1.000	0.439	3	1.461	0.061	3	1.462	0.071	1	1.183	-
	48	3	1.000	0.144	3	1.038	0.086	3	0.998	0.048	3	1.173	0.081
	72	3	1.000	0.034	3	1.007	0.062	3	1.018	0.080	3	1.220	0.083
62	0	3	1.200	0.156	3	1.272	0.129	3	1.245	0.084	3	1.312	0.140
	1	3	0.816	0.313	3	0.693	0.102	3	0.778	0.194	3	0.980	0.398
	2	3	1.069	0.097	3	0.850	0.187	2	1.070	0.067	3	1.123	0.111
	4	3	1.039	0.070	3	0.939	0.046	3	1.084	0.060	3	1.155	0.082
	8	3	1.022	0.188	3	1.024	0.224	3	0.974	0.363	3	1.331	0.170
	12	3	0.829	0.015	3	0.809	0.134	3	1.017[b]	0.100	3	0.989	0.184
	16	3	0.805	0.125	3	0.842	0.049	3	0.865	0.023	3	1.045[b]	0.036
	20	3	0.986	0.298	3	1.031	0.122	3	0.976	0.135	3	1.159	0.055
	24	3	0.952	0.179	3	0.961	0.120	3	0.949	0.185	3	1.335[b]	0.116
	48	3	0.849	0.196	3	0.768	0.158	3	0.679	0.138	3	0.934	0.149
	72	3	0.850	0.148	3	0.855	0.076	3	0.884	0.161	3	0.996	0.113

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-9. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	51.2	1.5	3	39.8	17.3	3	55.5	3.6	3	53.5	2.1
	1	3	33.2	4.0	3	38.0	3.3	3	39.0	4.5	3	37.6	2.4
	2	3	39.5	5.0	3	48.3	0.3	3	50.0	5.3	3	46.1	0.8
	4	3	58.8	0.4	3	58.5	4.1	3	60.3	0.6	3	51.3	6.7
	8	3	48.3	1.8	3	50.1	6.6	3	52.4	5.1	3	48.4	2.9
	12	3	60.6	7.5	3	61.1	7.0	3	68.6	5.8	3	58.8	7.2
	16	3	84.2	1.6	3	75.5	13.1	3	72.5	15.4	3	75.3	6.3
	20	3	68.4	2.2	3	78.1	8.0	3	74.0	10.6	3	75.2	8.2
	24	2	130.2	21.7	3	126.5	22.4	3	145.5	15.1	3	127.4	25.3
	48	2	187.6	27.6	1	294.4	-	1	335.2	-	2	314.8	21.8
	72	2	291.2	14.9	1	307.0	-	0	-	-	3	307.7	5.7
101	0	3	54.2	2.0	3	54.7	2.9	3	55.3	2.5	3	51.9	3.9
	1	2	32.7	2.8	3	43.9	1.9	3	36.7	10.1	3	43.1	8.0
	2	3	44.4	2.5	3	43.5	5.1	3	44.7	4.3	3	51.9	4.3
	4	3	54.6	6.0	3	57.0	1.6	3	59.8	3.4	3	52.0	9.6
	8	3	45.9	2.8	3	42.8	4.2	3	47.3	4.5	3	49.5	3.5
	12	3	43.3	0.8	3	40.2	8.4	3	39.7	13.9	3	61.2	4.0
	16	3	59.1	8.2	3	53.2	19.8	3	64.9	3.4	3	63.8	9.7
	20	3	47.8	9.0	3	46.3	2.1	2	51.0	3.6	3	48.9	5.8
	24	2	86.7	6.4	3	85.6	4.9	3	102.1	21.2	3	97.3	5.2
	48	3	113.2	9.6	2	121.4	49.4	3	102.1	32.0	3	124.9	21.3
	72	3	160.0	26.0	3	178.3	1.2	3	159.9	32.6	3	191.7	18.2

TABLE G-10. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.029	3	0.778	0.337	3	1.085	0.070	3	1.045	0.041
	1	3	1.000	0.120	3	1.146	0.100	3	1.176	0.135	3	1.136	0.073
	2	3	1.000	0.127	3	1.221	0.008	3	1.266	0.133	3	1.168	0.021
	4	3	1.000	0.008	3	0.996	0.070	3	1.026	0.011	3	0.873	0.115
	8	3	1.000	0.038	3	1.037	0.136	3	1.084	0.106	3	1.001	0.060
	12	3	1.000	0.123	3	1.007	0.115	3	1.131	0.095	3	0.969	0.118
	16	3	1.000	0.019	3	0.896	0.155	3	0.861	0.183	3	0.894	0.075
	20	3	1.000	0.032	3	1.142	0.117	3	1.081	0.155	3	1.099	0.119
	24	2	1.000	0.167	3	0.972	0.172	3	1.117	0.116	3	0.978	0.194
	48	2	1.000	0.147	1	1.569	-	1	1.787	-	2	1.678	0.116
	72	2	1.000	0.051	1	1.055	-	0	-	-	3	1.057	0.019
101	0	3	1.060	0.038	3	1.070	0.056	3	1.081	0.049	3	1.015	0.076
	1	2	0.988	0.083	3	1.323[b]	0.057	3	1.108	0.305	3	1.300[b]	0.241
	2	3	1.124	0.064	3	1.102	0.128	3	1.130	0.109	3	1.314	0.109
	4	3	0.929	0.103	3	0.970	0.028	3	1.019	0.057	3	0.885	0.163
	8	3	0.950	0.059	3	0.885	0.086	3	0.979	0.093	3	1.024	0.071
	12	3	0.714[a]	0.013	3	0.664	0.139	3	0.655	0.229	3	1.010[b]	0.066
	16	3	0.702[a]	0.098	3	0.632	0.236	3	0.770	0.040	3	0.757	0.115
	20	3	0.699[a]	0.131	3	0.676	0.031	2	0.745	0.053	3	0.715	0.085
	24	2	0.666[a]	0.049	3	0.658	0.038	3	0.784	0.163	3	0.748	0.040
	48	3	0.604[a]	0.051	2	0.647	0.263	3	0.544	0.170	3	0.666	0.113
	72	3	0.550[a]	0.089	3	0.612	0.004	3	0.549	0.112	3	0.658	0.062

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-11. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	55.3	1.8	3	55.9	3.5	3	53.6	1.6	3	53.7	3.2
	1	3	61.7	3.4	3	58.4	1.0	3	62.3	5.3	3	57.1	1.9
	2	3	40.7	2.1	3	43.0	1.2	3	45.8	3.0	3	55.6	5.5
	4	3	43.9	6.4	3	52.7	3.5	3	49.7	6.4	3	51.6	3.5
	8	3	56.3	6.6	3	49.9	20.0	3	65.9	3.2	3	62.2	18.3
	12	3	69.8	12.7	3	66.3	4.5	3	74.3	8.4	3	80.3	17.9
	16	3	83.7	6.9	3	95.0	14.1	3	103.8	8.5	3	101.9	10.6
	20	3	101.3	12.0	3	106.4	11.1	3	110.4	4.0	3	117.1	15.5
	24	3	91.6	9.5	3	103.4	10.5	3	83.1	7.6	3	99.2	13.4
	48	3	101.6	26.3	3	99.4	8.2	3	95.9	9.0	3	103.6	5.2
	72	2	86.5	2.7	3	99.4	8.2	3	95.9	9.0	3	103.6	5.2
101	0	3	59.3	13.1	3	61.5	7.1	3	63.9	5.6	3	58.4	8.7
	1	2	61.2	3.8	3	58.0	0.4	3	59.0	5.6	3	58.1	4.5
	2	3	42.3	4.7	3	38.6	5.7	3	40.7	4.6	3	55.5	5.1
	4	3	40.9	9.4	3	35.2	5.5	3	39.8	3.9	3	46.7	10.6
	8	3	38.7	7.6	3	34.8	10.9	3	52.3	8.2	3	46.5	4.1
	12	3	53.5	9.4	3	55.3	7.9	3	56.0	17.3	3	75.4	12.8
	16	3	45.3	5.8	3	47.9	10.5	3	54.4	5.9	3	73.9	4.7
	20	3	61.4	16.3	3	60.0	11.4	3	77.5	8.9	3	70.3	20.8
	24	3	44.4	6.6	3	43.5	16.0	3	56.7	5.1	2	58.2	12.5
	48	3	42.1	26.8	3	33.4	3.5	3	46.2	7.0	3	57.6	23.8
	72	3	42.1	26.8	3	33.4	3.5	3	46.2	7.0	3	57.6	23.8

TABLE G-12. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.032	3	1.012	0.064	3	0.969	0.029	3	0.972	0.058
	1	3	1.000	0.055	3	0.947	0.016	3	1.010	0.085	3	0.925	0.031
	2	3	1.000	0.051	3	1.057	0.030	3	1.126	0.073	3	1.366	0.135
	4	3	1.000	0.147	3	1.200	0.079	3	1.130	0.145	3	1.174	0.079
	8	3	1.000	0.117	3	0.887	0.355	3	1.171	0.056	3	1.106	0.326
	12	3	1.000	0.182	3	0.950	0.064	3	1.065	0.120	3	1.151	0.257
	16	3	1.000	0.083	3	1.134	0.168	3	1.239	0.101	3	1.217	0.127
	20	3	1.000	0.118	3	1.051	0.110	3	1.090	0.039	3	1.156	0.153
	24	3	1.000	0.104	3	1.128	0.114	3	0.907	0.083	3	1.082	0.146
	48	3	1.000	0.259	3	0.978	0.080	3	0.943	0.088	3	1.020	0.052
	72	2	1.000	0.031	3	1.150	0.095	3	1.109	0.104	3	1.198	0.061
101	0	3	1.074	0.236	3	1.113	0.129	3	1.157	0.100	3	1.056	0.158
	1	2	0.993	0.062	3	0.940	0.007	3	0.957	0.091	3	0.943	0.073
	2	3	1.040	0.116	3	0.950	0.139	3	1.000	0.112	3	1.364[b]	0.125
	4	3	0.931	0.214	3	0.800	0.125	3	0.905	0.088	3	1.062	0.240
	8	3	0.687[a]	0.135	3	0.619	0.194	3	0.930[b]	0.146	3	0.827	0.073
	12	3	0.766	0.135	3	0.793	0.114	3	0.803	0.248	3	1.081[b]	0.183
	16	3	0.541[a]	0.069	3	0.572	0.125	3	0.650	0.070	3	0.883[b]	0.056
	20	3	0.607[a]	0.161	3	0.592	0.112	3	0.766	0.088	3	0.694	0.206
	24	3	0.485[a]	0.072	3	0.474	0.174	3	0.618	0.056	2	0.635	0.137
	48	3	0.414[a]	0.264	3	0.329	0.035	3	0.454	0.069	3	0.566	0.234
	72	3	0.487[a]	0.310	3	0.386	0.041	3	0.534	0.081	3	0.666	0.275

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-13. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	30.2	4.5	3	32.6	1.7	3	33.3	0.8	3	32.4	1.9
	2	3	26.4	5.3	3	34.4	1.2	3	33.8	3.4	3	35.8	5.5
	4	3	42.8	3.2	3	35.8	2.3	3	37.4	1.7	3	36.1	2.0
	8	3	56.8	1.1	3	54.6	3.8	3	55.4	5.9	3	52.2	3.5
	12	3	61.5	5.2	3	62.7	3.7	3	67.9	2.2	3	68.7	4.2
	16	3	69.0	3.8	3	63.7	7.7	3	70.2	13.2	3	73.8	3.6
	20	3	91.1	11.2	3	101.5	2.3	3	90.5	14.7	3	88.6	8.0
	24	3	124.7	8.5	3	129.1	35.0	3	91.1	45.2	3	120.1	3.8
	48	2	138.7	6.9	3	140.7	28.6	3	159.5	17.4	3	167.5	10.3
	72	3	214.1	68.5	3	269.3	10.4	3	241.7	37.8	3	244.4	25.5
171	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	32.8	3.4	3	29.0	2.0	3	28.5	4.0	3	34.7	3.1
	2	3	29.0	9.8	3	32.9	4.0	3	35.0	6.4	3	38.2	9.6
	4	3	39.1	4.3	3	32.7	1.9	3	35.1	3.2	3	40.7	0.9
	8	3	43.5	9.1	3	38.2	3.7	3	40.6	1.3	3	54.3	11.1
	12	3	36.0	8.6	3	31.6	1.9	3	37.5	3.3	3	46.9	6.9
	16	3	34.4	4.1	3	34.0	1.7	3	27.0	8.4	3	45.6	11.8
	20	3	30.2	0.6	3	31.8	3.4	3	33.6	7.2	3	50.2	0.4
	24	3	40.0	1.5	3	32.7	5.1	3	34.9	9.6	3	50.9	4.4
	48	3	12.3	3.6	3	12.7	2.5	3	17.8	2.9	3	24.2	3.1
	72	3	13.0	1.2	3	9.1	0.4	3	12.3	1.1	3	14.7	2.6

TABLE G-14. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	1.000	0.150	3	1.079	0.056	3	1.105	0.028	3	1.073	0.061
	2	3	1.000	0.199	3	1.300	0.046	3	1.277	0.128	3	1.355	0.209
	4	3	1.000	0.076	3	0.836	0.055	3	0.874	0.041	3	0.844	0.046
	8	3	1.000	0.019	3	0.961	0.067	3	0.975	0.104	3	0.920	0.062
	12	3	1.000	0.084	3	1.020	0.061	3	1.105	0.035	3	1.118	0.068
	16	3	1.000	0.055	3	0.924	0.111	3	1.018	0.191	3	1.070	0.053
	20	3	1.000	0.123	3	1.115	0.025	3	0.993	0.161	3	0.972	0.088
	24	3	1.000	0.068	3	1.035	0.281	3	0.730	0.362	3	0.963	0.031
	48	2	1.000	0.050	3	1.014	0.206	3	1.150	0.126	3	1.207	0.074
	72	3	1.000	0.320	3	1.258	0.049	3	1.129	0.177	3	1.141	0.119
171	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	1.088	0.112	3	0.962	0.068	3	0.944	0.131	3	1.150	0.102
	2	3	1.098	0.370	3	1.244	0.152	3	1.322	0.242	3	1.445	0.364
	4	3	0.913	0.100	3	0.764	0.044	3	0.820	0.075	3	0.950	0.021
	8	3	0.766	0.160	3	0.672	0.066	3	0.715	0.023	3	0.956	0.195
	12	3	0.585[a]	0.140	3	0.513	0.032	3	0.610	0.054	3	0.763[b]	0.112
	16	3	0.499[a]	0.059	3	0.492	0.024	3	0.391	0.122	3	0.661	0.172
	20	3	0.331[a]	0.006	3	0.349	0.037	3	0.369	0.079	3	0.551[b]	0.004
	24	3	0.320[a]	0.012	3	0.262	0.041	3	0.280	0.077	3	0.408	0.035
	48	3	0.089[a]	0.026	3	0.091	0.018	3	0.128	0.021	3	0.174[b]	0.022
	72	3	0.061[a]	0.006	3	0.043	0.002	3	0.057	0.005	3	0.069	0.012

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.

[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

TABLE G-15. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	58.7	1.4	3	54.9	8.7	3	54.9	2.2	3	47.9	1.9
	1	3	54.5	4.2	3	52.9	1.0	3	51.9	3.5	3	50.0	2.0
	2	3	39.5	4.9	3	40.1	2.8	3	44.1	2.1	3	46.2	3.2
	4	3	45.9	1.1	3	44.3	8.8	3	55.7	11.9	3	53.8	2.0
	8	3	58.5	6.4	3	61.3	7.7	3	54.3	5.8	3	69.7	5.1
	12	3	81.1	10.1	3	83.8	7.7	3	92.4	5.8	3	103.5	18.0
	16	3	100.5	5.1	3	103.3	1.9	3	106.2	11.8	3	111.6	16.9
	20	3	73.4	11.3	3	78.7	12.1	3	92.8	4.5	3	98.0	17.5
	24	3	71.7	3.4	3	69.1	4.7	3	79.7	9.9	3	79.8	4.2
	48	3	88.9	7.3	3	97.1	13.1	3	107.2	9.4	3	114.6	7.9
	72	3	111.8	19.7	3	101.2	2.9	3	106.4	10.0	3	119.2	12.7
171	0	3	65.3	1.8	3	63.6	5.3	3	66.3	0.4	3	66.4	5.4
	1	3	52.0	11.5	3	46.7	7.2	3	45.9	4.9	3	50.5	1.6
	2	3	38.2	6.1	3	37.6	3.1	3	34.9	8.3	3	42.5	8.9
	4	3	43.8	10.7	3	32.1	3.8	3	38.6	5.4	3	49.3	9.6
	8	3	29.8	3.1	3	35.8	10.5	3	19.9	4.5	3	47.1	5.8
	12	3	43.1	11.2	3	34.9	2.7	3	47.5	6.8	3	59.6	8.5
	16	3	33.9	5.2	3	36.9	4.6	3	42.8	3.1	3	62.2	13.1
	20	3	37.2	9.3	3	33.5	1.7	3	44.6	1.6	3	52.2	9.2
	24	3	22.5	5.9	3	19.0	1.2	3	25.9	5.5	3	38.3	8.2
	48	3	14.6	3.1	3	12.6	3.0	3	18.0	0.9	3	17.4	6.7
	72	3	8.5	1.2	3	7.4	1.6	3	8.4	0.7	3	14.3	2.0

TABLE G-16. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.024	3	0.935	0.148	3	0.936	0.038	3	0.816	0.032
	1	3	1.000	0.077	3	0.970	0.019	3	0.951	0.064	3	0.917	0.036
	2	3	1.000	0.123	3	1.014	0.070	3	1.116	0.054	3	1.170	0.081
	4	3	1.000	0.024	3	0.964	0.192	3	1.214	0.260	3	1.172	0.044
	8	3	1.000	0.109	3	1.048	0.131	3	0.928	0.100	3	1.192	0.087
	12	3	1.000	0.124	3	1.033	0.095	3	1.139	0.072	3	1.275	0.222
	16	3	1.000	0.051	3	1.029	0.019	3	1.057	0.118	3	1.111	0.168
	20	3	1.000	0.153	3	1.071	0.164	3	1.263	0.062	3	1.335	0.238
	24	3	1.000	0.047	3	0.965	0.065	3	1.113	0.138	3	1.113	0.058
	48	3	1.000	0.082	3	1.092	0.147	3	1.206	0.105	3	1.289	0.089
	72	3	1.000	0.176	3	0.905	0.026	3	0.952	0.089	3	1.066	0.114
171	0	3	1.113	0.030	3	1.084	0.090	3	1.130	0.007	3	1.131	0.092
	1	3	0.954	0.210	3	0.856	0.132	3	0.842	0.090	3	0.925	0.030
	2	3	0.968	0.154	3	0.951	0.080	3	0.882	0.209	3	1.077	0.225
	4	3	0.955	0.234	3	0.700[b]	0.083	3	0.841	0.118	3	1.073	0.208
	8	3	0.509[a]	0.052	3	0.612	0.180	3	0.340	0.077	3	0.806[b]	0.099
	12	3	0.531[a]	0.138	3	0.431	0.033	3	0.586	0.084	3	0.735[b]	0.105
	16	3	0.338[a]	0.051	3	0.367	0.046	3	0.426	0.031	3	0.619[b]	0.130
	20	3	0.507[a]	0.127	3	0.457	0.023	3	0.608	0.022	3	0.711[b]	0.125
	24	3	0.314[a]	0.082	3	0.265	0.017	3	0.361	0.077	3	0.535[b]	0.115
	48	3	0.164[a]	0.035	3	0.142	0.034	3	0.203	0.010	3	0.196	0.076
	72	3	0.076[a]	0.010	3	0.067	0.014	3	0.075	0.006	3	0.128[b]	0.018

[a]Differs ($p \leq 0.05$) from the vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from the HD-control (HD-exposed, NM = 0) value.

APPENDIX H

SUMMARY STATISTICS FOR NAD^+ DATA FROM NIACIN-PRETREATED, HD-EXPOSED CULTURES

TABLE H-1. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	28.5	0.7	2	28.2	3.3	3	22.0	17.0	2	32.3	1.0
	2	3	31.1	5.1	3	27.9	4.3	3	32.4	3.5	3	31.5	2.1
	4	3	28.9	4.4	3	31.7	0.4	3	28.9	2.4	3	29.8	0.8
	8	3	42.2	1.7	3	38.7	3.2	3	36.6	2.1	3	34.7	1.1
	12	3	63.1	5.0	3	45.8	5.0	3	39.9	2.3	3	36.5	3.7
	16	3	54.7	15.0	3	43.0	2.7	3	35.8	5.3	3	40.2	3.6
	20	3	47.5	8.0	3	52.6	4.7	3	48.4	4.1	3	45.8	4.3
	24	3	53.1	14.3	3	36.8	13.1	3	35.9	4.2	3	34.8	2.4
	48	3	91.4	6.3	3	85.8	13.6	3	69.4	12.2	3	67.1	1.2
	72	3	131.4	8.6	3	131.7	16.3	3	122.2	15.9	3	93.3	10.6
13	0	3	29.2	9.1	3	29.3	4.1	3	25.7	2.0	3	30.7	4.2
	2	3	32.9	6.0	3	30.8	2.3	3	32.2	3.6	3	33.7	0.6
	4	3	28.8	3.8	3	23.6	12.2	3	25.8	1.4	2	24.8	7.9
	8	3	49.3	5.0	3	35.3	5.4	3	33.4	3.3	3	32.8	5.7
	12	3	57.3	6.8	3	42.2	5.6	3	37.8	0.5	3	34.2	6.6
	16	3	50.7	3.2	3	44.5	5.7	3	32.4	4.8	3	30.5	4.4
	20	3	59.0	9.0	3	56.3	13.4	3	45.5	10.7	3	50.3	4.4
	24	3	39.9	10.3	3	33.6	13.5	2	32.7	1.1	2	27.8	0.0
	48	2	99.2	16.7	2	71.5	4.0	2	62.4	4.4	2	58.1	10.1
	72	2	124.5	14.7	2	107.2	9.7	2	77.2	14.6	2	73.2	18.1

TABLE H-2. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	1.000	0.025	2	0.987	0.115	3	0.770	0.596	2	1.133	0.036
	2	3	1.000	0.163	3	0.896	0.139	3	1.041	0.114	3	1.012	0.068
	4	3	1.000	0.152	3	1.095	0.013	3	0.997	0.084	3	1.029	0.029
	8	3	1.000	0.040	3	0.917	0.076	3	0.869	0.050	3	0.823	0.025
	12	3	1.000	0.080	3	0.725	0.079	3	0.632	0.036	3	0.579	0.058
	16	3	1.000	0.275	3	0.786	0.049	3	0.655	0.098	3	0.735	0.066
	20	3	1.000	0.167	3	1.108	0.099	3	1.019	0.087	3	0.965	0.091
	24	3	1.000	0.270	3	0.693	0.248	3	0.676	0.079	3	0.657	0.046
	48	3	1.000	0.069	3	0.939	0.148	3	0.760	0.134	3	0.734	0.014
	72	3	1.000	0.066	3	1.003	0.124	3	0.930	0.121	3	0.710	0.081
13	0	3	1.023	0.319	3	1.026	0.143	3	0.899	0.070	3	1.076	0.146
	2	3	1.056	0.192	3	0.991	0.075	3	1.036	0.116	3	1.083	0.019
	4	3	0.997	0.132	3	0.815	0.423	3	0.894	0.050	2	0.858	0.274
	8	3	1.169	0.118	3	0.837[b]	0.129	3	0.792[b]	0.078	3	0.777[b]	0.136
	12	3	0.907	0.107	3	0.669[b]	0.088	3	0.598[b]	0.008	3	0.541[b]	0.104
	16	3	0.926	0.058	3	0.815	0.104	3	0.593[b]	0.089	3	0.557[b]	0.080
	20	3	1.241	0.189	3	1.184	0.281	3	0.959	0.225	3	1.060	0.092
	24	3	0.751	0.194	3	0.633	0.255	2	0.616	0.021	2	0.524	0.000
	48	2	1.085	0.182	2	0.783[b]	0.044	2	0.683[b]	0.048	2	0.636[b]	0.110
	72	2	0.948	0.112	2	0.816	0.074	2	0.588[b]	0.111	2	0.557[b]	0.138

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-3. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	66.5	21.6	3	74.8	3.5	3	79.7	4.2	3	83.6	4.2
	1	3	41.8	5.5	3	42.2	4.2	3	45.4	3.6	3	41.9	3.7
	2	3	39.8	8.2	3	38.4	8.6	3	40.7	1.3	3	40.1	5.5
	4	3	61.4	2.5	3	57.5	5.4	3	55.5	5.2	3	66.6	6.4
	8	3	78.9	15.9	3	74.4	21.7	3	66.8	8.6	3	64.2	4.0
	12	3	73.5	23.5	3	91.1	20.2	3	80.1	3.1	3	67.2	16.5
	16	3	89.6	16.8	3	116.6	10.4	3	99.1	4.0	3	85.3	3.0
	20	3	93.9	11.3	3	106.5	13.3	3	91.6	6.6	3	77.9	17.6
	24	3	110.8	17.9	3	105.1	7.9	3	140.5	4.4	3	121.4	20.3
	48	3	116.5	9.2	3	143.0	9.7	3	114.2	9.8	3	110.2	13.5
	72	3	129.0	6.1	3	140.6	9.1	3	145.5	6.6	3	118.9	12.5
13	0	3	83.2	6.3	3	84.8	3.5	3	85.9	1.9	3	91.1	2.4
	1	3	46.7	2.2	3	48.5	3.6	3	47.3	5.1	3	45.4	6.4
	2	3	41.3	7.9	3	36.9	6.4	3	36.3	2.9	3	35.7	5.5
	4	3	68.2	8.0	3	64.3	11.2	3	53.4	8.9	3	74.3	12.4
	8	3	74.2	15.2	3	76.9	10.6	3	60.4	4.4	3	62.4	9.9
	12	3	99.7	21.1	3	90.1	11.5	3	73.4	6.9	3	66.8	3.1
	16	3	82.9	13.6	3	100.0	4.0	3	83.3	7.6	3	90.8	6.3
	20	3	98.1	25.4	3	110.7	20.2	2	81.6	3.6	3	82.7	22.3
	24	3	106.0	5.0	3	102.9	16.6	3	131.0	8.7	3	106.3	4.4
	48	3	101.1	8.0	3	154.1	1.7	3	115.3	11.2	3	88.9	8.1
	72	3	121.1	29.0	3	164.9	11.7	3	149.8	12.4	3	110.4	7.8

TABLE H-4. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.325	3	1.125	0.052	3	1.198	0.063	3	1.257	0.062
	1	3	1.000	0.131	3	1.011	0.101	3	1.088	0.085	3	1.003	0.089
	2	3	1.000	0.205	3	0.966	0.215	3	1.023	0.033	3	1.007	0.138
	4	3	1.000	0.040	3	0.936	0.088	3	0.903	0.084	3	1.086	0.104
	8	3	1.000	0.201	3	0.944	0.276	3	0.847	0.109	3	0.814	0.051
	12	3	1.000	0.320	3	1.240	0.275	3	1.089	0.042	3	0.914	0.225
	16	3	1.000	0.187	3	1.301	0.116	3	1.106	0.045	3	0.952	0.034
	20	3	1.000	0.120	3	1.134	0.141	3	0.976	0.071	3	0.829	0.188
	24	3	1.000	0.161	3	0.948	0.071	3	1.268	0.039	3	1.095	0.184
	48	3	1.000	0.079	3	1.227	0.083	3	0.980	0.084	3	0.945	0.116
	72	3	1.000	0.047	3	1.090	0.071	3	1.128	0.051	3	0.922	0.097
13	0	3	1.251	0.094	3	1.274	0.052	3	1.292	0.028	3	1.369	0.036
	1	3	1.119	0.051	3	1.162	0.086	3	1.133	0.123	3	1.087	0.154
	2	3	1.038	0.199	3	0.926	0.160	3	0.912	0.073	3	0.898	0.139
	4	3	1.111	0.130	3	1.048	0.182	3	0.870	0.146	3	1.210	0.202
	8	3	0.940	0.192	3	0.975	0.134	3	0.766	0.056	3	0.791	0.126
	12	3	1.356[a]	0.287	3	1.226	0.156	3	0.999[b]	0.094	3	0.909[b]	0.043
	16	3	0.925	0.152	3	1.115[b]	0.044	3	0.929	0.085	3	1.013	0.070
	20	3	1.045	0.270	3	1.179	0.215	2	0.869	0.038	3	0.881	0.237
	24	3	0.957	0.045	3	0.928	0.149	3	1.182	0.079	3	0.959	0.039
	48	3	0.868	0.069	3	1.322[b]	0.014	3	0.989	0.096	3	0.763	0.070
	72	3	0.939	0.225	3	1.279[b]	0.091	3	1.161	0.096	3	0.856	0.061

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-5. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	44.8	3.9	3	42.3	4.7	2	42.1	2.2	3	42.6	5.8
	1	3	41.7	8.0	3	34.3	1.4	3	33.0	8.6	2	39.3	0.6
	2	3	39.4	11.9	3	40.8	2.1	3	40.9	1.5	3	38.7	0.9
	4	3	36.4	4.2	3	35.1	1.3	3	35.1	1.6	3	31.0	1.2
	8	3	48.4	5.2	3	45.0	1.4	3	40.3	1.5	3	39.8	2.9
	12	3	53.8	2.8	3	50.4	4.0	3	39.6	1.6	3	38.4	1.5
	16	3	76.7	10.5	3	52.7	4.4	3	40.7	4.2	3	39.5	1.1
	20	3	47.4	6.8	3	54.3	4.8	3	43.1	3.9	3	45.5	2.9
	24	3	55.8	8.9	3	57.1	5.1	3	46.8	7.8	3	50.5	2.7
	48	2	85.0	1.9	3	77.1	18.4	3	64.4	3.5	3	57.6	4.4
	72	3	187.6	19.5	3	168.0	25.3	3	151.2	6.8	3	117.8	3.2
62	0	1	43.6	-	2	47.0	0.5	2	48.2	1.6	2	45.3	4.8
	1	3	45.3	6.7	3	33.7	2.1	3	38.6	0.8	2	46.4	7.2
	2	3	37.1	10.8	2	41.3	2.7	3	38.0	6.3	3	38.6	6.4
	4	3	35.4	5.5	3	33.4	2.9	3	30.9	3.4	3	31.3	3.5
	8	3	45.9	3.7	3	39.1	7.5	3	38.0	4.2	3	37.1	3.2
	12	3	57.2	2.7	3	46.6	7.0	3	36.9	5.3	3	37.9	2.9
	16	3	60.2	3.6	3	43.2	4.0	3	36.4	2.4	3	31.5	4.8
	20	3	57.5	13.0	3	51.7	5.8	3	39.0	3.6	3	43.3	2.6
	24	3	47.8	9.7	3	52.6	1.8	3	25.0	20.5	2	47.5	6.1
	48	2	62.0	10.9	3	55.4	10.7	3	46.4	7.3	3	43.1	11.6
	72	3	123.3	8.6	3	90.6	5.1	3	78.5	3.5	3	74.7	14.9

TABLE H-6. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.087	3	0.945	0.104	2	0.941	0.048	3	0.951	0.130
	1	3	1.000	0.192	3	0.823	0.033	3	0.791	0.206	2	0.944	0.015
	2	3	1.000	0.303	3	1.036	0.052	3	1.038	0.037	3	0.984	0.022
	4	3	1.000	0.115	3	0.965	0.034	3	0.965	0.043	3	0.851	0.034
	8	3	1.000	0.107	3	0.931	0.030	3	0.832	0.031	3	0.823	0.060
	12	3	1.000	0.052	3	0.937	0.075	3	0.736	0.030	3	0.713	0.027
	16	3	1.000	0.137	3	0.688	0.057	3	0.530	0.054	3	0.514	0.014
	20	3	1.000	0.144	3	1.147	0.100	3	0.910	0.081	3	0.961	0.061
	24	3	1.000	0.160	3	1.023	0.091	3	0.837	0.139	3	0.904	0.048
	48	2	1.000	0.022	3	0.908	0.217	3	0.758	0.042	3	0.678	0.051
	72	3	1.000	0.104	3	0.896	0.135	3	0.806	0.036	3	0.628	0.017
62	0	1	0.974	-	2	1.050	0.011	2	1.076	0.037	2	1.011	0.108
	1	3	1.086	0.160	3	0.808	0.051	3	0.927	0.019	2	1.115	0.173
	2	3	0.943	0.274	2	1.049	0.068	3	0.964	0.159	3	0.980	0.164
	4	3	0.972	0.150	3	0.918	0.080	3	0.849	0.093	3	0.859	0.095
	8	3	0.948	0.076	3	0.807	0.156	3	0.785	0.088	3	0.766	0.066
	12	3	1.063	0.051	3	0.867	0.130	3	0.685[b]	0.098	3	0.704[b]	0.054
	16	3	0.784	0.047	3	0.564[b]	0.052	3	0.475[b]	0.031	3	0.411[b]	0.063
	20	3	1.213	0.274	3	1.091	0.122	3	0.824[b]	0.075	3	0.914	0.054
	24	3	0.857	0.173	3	0.941	0.032	3	0.448[b]	0.367	2	0.851	0.109
	48	2	0.730	0.128	3	0.651	0.125	3	0.546	0.086	3	0.508	0.136
	72	3	0.658[a]	0.046	3	0.483[b]	0.027	3	0.419[b]	0.018	3	0.398[b]	0.079

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-7. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	54.9	8.0	3	61.1	3.6	2	61.3	9.7	3	68.0	2.5
	1	3	46.0	3.3	3	46.7	6.9	3	49.6	2.9	3	49.7	0.9
	2	3	54.5	2.7	3	56.2	13.2	3	58.7	4.2	3	54.7	4.6
	4	3	71.9	7.9	3	72.8	16.9	3	76.6	1.7	3	66.0	9.0
	8	3	68.2	10.0	3	75.2	9.6	3	68.5	9.3	3	68.9	3.3
	12	3	104.2	19.5	3	107.8	10.5	3	133.4	8.2	3	77.8	1.6
	16	3	116.7	7.1	3	107.8	10.6	3	99.1	3.8	3	86.5	7.7
	20	3	107.5	26.1	3	129.3	9.9	3	107.3	6.8	3	93.2	8.1
	24	3	96.7	42.4	1	141.2	-	3	111.5	14.3	3	110.0	4.4
	48	3	145.0	20.8	3	144.4	22.4	3	132.4	10.4	3	113.0	8.7
	72	3	167.7	5.6	3	165.8	17.1	3	172.3	12.3	3	142.1	14.9
62	0	3	65.8	8.5	3	63.3	3.6	3	74.7	3.8	3	71.5	8.3
	1	3	37.5	14.4	3	39.7	13.8	3	39.1	12.7	3	46.2	17.5
	2	3	58.3	5.3	3	59.0	7.3	3	56.0	2.8	3	54.3	6.1
	4	3	74.7	5.0	3	78.6	8.7	3	66.5	3.5	3	63.6	10.2
	8	3	69.7	12.8	3	75.5	13.3	3	59.3	9.9	3	66.0	10.1
	12	3	86.4	1.6	3	64.7	54.3	3	115.1	13.1	3	75.9	6.9
	16	3	93.9	14.6	3	105.2	6.0	3	81.7	5.1	3	82.0	5.5
	20	3	106.0	32.1	3	106.4	16.9	3	89.4	14.3	3	81.0	14.2
	24	3	92.1	17.3	3	113.5	21.4	3	80.3	8.6	3	88.8	17.3
	48	3	123.1	28.4	3	121.9	13.7	3	95.7	18.8	3	89.9	8.9
	72	3	142.5	24.8	3	128.8	7.2	3	133.0	9.3	3	107.7	12.3

TABLE H-8. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.146	3	1.114	0.065	2	1.117	0.177	3	1.240	0.045
	1	3	1.000	0.071	3	1.016	0.151	3	1.079	0.063	3	1.082	0.019
	2	3	1.000	0.049	3	1.030	0.243	3	1.076	0.077	3	1.003	0.085
	4	3	1.000	0.109	3	1.013	0.234	3	1.065	0.024	3	0.918	0.125
	8	3	1.000	0.146	3	1.103	0.141	3	1.004	0.136	3	1.010	0.049
	12	3	1.000	0.187	3	1.035	0.101	3	1.281	0.078	3	0.747	0.015
	16	3	1.000	0.061	3	0.924	0.091	3	0.849	0.032	3	0.741	0.066
	20	3	1.000	0.243	3	1.202	0.092	3	0.998	0.063	3	0.867	0.075
	24	3	1.000	0.439	1	1.461	-	3	1.154	0.148	3	1.137	0.046
	48	3	1.000	0.144	3	0.995	0.154	3	0.913	0.072	3	0.779	0.060
	72	3	1.000	0.034	3	0.989	0.102	3	1.027	0.073	3	0.848	0.089
62	0	3	1.200	0.156	3	1.155	0.065	3	1.361	0.069	3	1.303	0.151
	1	3	0.816	0.313	3	0.864	0.301	3	0.850	0.277	3	1.004	0.380
	2	3	1.069	0.097	3	1.081	0.135	3	1.027	0.052	3	0.995	0.111
	4	3	1.039	0.070	3	1.093	0.121	3	0.926	0.049	3	0.885	0.142
	8	3	1.022	0.188	3	1.108	0.195	3	0.869	0.145	3	0.967	0.149
	12	3	0.829	0.015	3	0.621	0.522	3	1.105	0.125	3	0.729	0.066
	16	3	0.805	0.125	3	0.901	0.052	3	0.700	0.044	3	0.702	0.047
	20	3	0.986	0.298	3	0.989	0.157	3	0.831	0.133	3	0.754	0.132
	24	3	0.952	0.179	3	1.174	0.222	3	0.831	0.088	3	0.919	0.179
	48	3	0.849	0.196	3	0.841	0.094	3	0.660	0.130	3	0.620[b]	0.061
	72	3	0.850	0.148	3	0.768	0.043	3	0.793	0.056	3	0.642[b]	0.073

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-9. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	51.2	1.5	3	43.5	5.3	3	52.3	5.6	3	46.0	10.0
	1	3	33.2	4.0	3	40.2	4.3	3	31.2	1.7	3	42.9	5.1
	2	3	39.5	5.0	3	40.2	5.2	3	43.5	3.7	3	47.1	3.7
	4	3	58.8	0.4	3	51.7	3.1	3	49.4	5.0	3	55.4	3.7
	8	3	48.3	1.8	3	41.2	5.0	3	37.2	1.3	3	36.5	3.0
	12	3	60.6	7.5	3	42.5	8.0	3	37.3	9.5	3	47.4	1.6
	16	3	84.2	1.6	3	63.2	7.1	3	56.2	1.3	3	58.2	6.4
	20	3	68.4	2.2	3	58.7	5.7	3	53.0	7.8	3	53.8	3.4
	24	2	130.2	21.7	3	133.7	13.4	3	94.2	16.7	3	86.7	7.1
	48	2	187.6	27.6	2	278.9	54.2	3	294.6	7.1	3	230.8	10.3
	72	2	291.2	14.9	3	276.8	17.7	3	255.8	29.7	3	226.9	16.9
101	0	3	54.2	2.0	3	51.8	0.8	3	51.6	2.6	3	55.2	0.4
	1	2	32.7	2.8	3	42.0	6.7	3	40.0	7.3	3	38.1	2.5
	2	3	44.4	2.5	3	41.1	1.9	3	38.7	3.7	3	42.9	6.0
	4	3	54.6	6.0	3	49.9	5.9	3	48.9	5.5	3	52.3	4.8
	8	3	45.9	2.8	3	41.6	2.5	3	33.9	2.7	3	39.4	4.7
	12	3	43.3	0.8	3	42.5	4.6	3	32.0	4.2	3	37.7	3.1
	16	3	59.1	8.2	3	50.7	6.9	3	38.7	3.0	3	46.6	4.3
	20	3	47.8	9.0	3	42.9	4.9	3	39.0	4.5	3	39.0	6.4
	24	2	86.7	6.4	3	75.0	17.9	3	60.2	14.1	3	61.1	8.4
	48	3	113.2	9.6	3	97.0	39.3	3	95.6	16.0	3	92.6	19.7
	72	3	160.0	26.0	3	168.4	24.0	3	123.4	19.9	3	133.0	25.1

TABLE H-10. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.029	3	0.850	0.104	3	1.022	0.110	3	0.900	0.196
	1	3	1.000	0.120	3	1.213	0.130	3	0.942	0.051	3	1.295	0.153
	2	3	1.000	0.127	3	1.019	0.132	3	1.101	0.093	3	1.193	0.095
	4	3	1.000	0.008	3	0.880	0.053	3	0.840	0.085	3	0.943	0.063
	8	3	1.000	0.038	3	0.852	0.103	3	0.770	0.028	3	0.755	0.063
	12	3	1.000	0.123	3	0.702	0.133	3	0.615	0.157	3	0.782	0.026
	16	3	1.000	0.019	3	0.751	0.084	3	0.667	0.016	3	0.691	0.077
	20	3	1.000	0.032	3	0.857	0.083	3	0.775	0.114	3	0.785	0.049
	24	2	1.000	0.167	3	1.027	0.103	3	0.723	0.128	3	0.666	0.055
	48	2	1.000	0.147	2	1.487	0.289	3	1.570	0.038	3	1.230	0.055
	72	2	1.000	0.051	3	0.951	0.061	3	0.879	0.102	3	0.779	0.058
101	0	3	1.060	0.038	3	1.013	0.015	3	1.009	0.051	3	1.079	0.007
	1	2	0.988	0.083	3	1.267	0.202	3	1.208	0.220	3	1.149	0.076
	2	3	1.124	0.064	3	1.041	0.049	3	0.978	0.093	3	1.087	0.151
	4	3	0.929	0.103	3	0.849	0.100	3	0.833	0.094	3	0.890	0.082
	8	3	0.950	0.059	3	0.861	0.051	3	0.702[b]	0.055	3	0.815	0.097
	12	3	0.714[a]	0.013	3	0.702	0.075	3	0.528[b]	0.069	3	0.622	0.052
	16	3	0.702[a]	0.098	3	0.602	0.082	3	0.459[b]	0.036	3	0.553[b]	0.052
	20	3	0.699[a]	0.131	3	0.626	0.072	3	0.569	0.066	3	0.569	0.093
	24	2	0.666[a]	0.049	3	0.576	0.138	3	0.463[b]	0.109	3	0.469[b]	0.065
	48	3	0.604[a]	0.051	3	0.517	0.210	3	0.510	0.085	3	0.494	0.105
	72	3	0.550[a]	0.089	3	0.578	0.082	3	0.424	0.068	3	0.457	0.086

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-11. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	55.3	1.8	3	53.6	3.1	3	51.5	3.7	3	57.4	0.6
	1	3	61.7	3.4	3	58.1	1.6	3	58.1	2.8	3	63.8	2.1
	2	3	40.7	2.1	3	55.2	7.6	3	52.6	2.7	3	51.9	6.3
	4	3	43.9	6.4	3	44.3	5.7	3	43.4	2.2	3	45.4	0.9
	8	3	56.3	6.6	3	52.2	5.1	3	42.8	5.8	3	46.0	10.4
	12	3	69.8	12.7	3	75.5	5.1	3	60.4	4.8	3	55.8	10.8
	16	3	83.7	6.9	3	89.6	5.7	3	80.9	4.8	3	74.7	4.8
	20	3	101.3	12.0	3	96.4	16.6	3	86.5	3.9	3	75.0	11.0
	24	3	91.6	9.5	3	83.4	5.1	3	79.0	5.7	3	66.8	5.7
	48	3	101.6	26.3	3	86.7	2.1	3	85.9	11.2	3	63.2	2.4
	72	2	86.5	2.7	3	86.7	2.1	3	85.9	11.2	3	63.2	2.4
101	0	3	59.3	13.1	3	56.9	2.4	3	61.3	9.2	3	64.2	12.1
	1	2	61.2	3.8	3	61.0	5.6	3	62.0	11.2	3	59.9	5.0
	2	3	42.3	4.7	3	57.3	4.8	3	49.9	3.5	3	56.5	5.9
	4	3	40.9	9.4	3	40.3	9.6	3	37.7	1.4	3	38.7	2.5
	8	3	38.7	7.6	3	47.4	8.6	3	36.2	5.5	3	41.8	9.7
	12	3	53.5	9.4	3	60.5	9.2	3	47.2	5.1	3	48.4	8.9
	16	3	45.3	5.8	3	59.4	6.8	3	47.4	8.7	3	46.5	8.3
	20	3	61.4	16.3	3	61.6	15.8	3	50.8	13.0	3	52.0	16.7
	24	3	44.4	6.6	3	50.2	3.0	3	42.9	6.8	3	33.4	0.1
	48	3	42.1	26.8	3	40.1	13.1	3	35.1	3.2	3	40.9	1.9
	72	3	42.1	26.8	3	40.1	13.1	3	35.1	3.2	3	40.9	1.9

TABLE H-12. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.032	3	0.969	0.056	3	0.932	0.068	3	1.038	0.010
	1	3	1.000	0.055	3	0.942	0.026	3	0.943	0.045	3	1.034	0.034
	2	3	1.000	0.051	3	1.356	0.186	3	1.294	0.067	3	1.277	0.155
	4	3	1.000	0.147	3	1.008	0.131	3	0.988	0.050	3	1.032	0.021
	8	3	1.000	0.117	3	0.927	0.091	3	0.761	0.104	3	0.818	0.186
	12	3	1.000	0.182	3	1.082	0.073	3	0.866	0.069	3	0.799	0.155
	16	3	1.000	0.083	3	1.071	0.067	3	0.966	0.058	3	0.892	0.058
	20	3	1.000	0.118	3	0.952	0.163	3	0.855	0.038	3	0.741	0.108
	24	3	1.000	0.104	3	0.910	0.056	3	0.862	0.062	3	0.729	0.062
	48	3	1.000	0.259	3	0.853	0.021	3	0.845	0.110	3	0.621	0.024
	72	2	1.000	0.031	3	1.002	0.025	3	0.993	0.129	3	0.730	0.028
101	0	3	1.074	0.236	3	1.030	0.044	3	1.110	0.166	3	1.161	0.219
	1	2	0.993	0.062	3	0.990	0.090	3	1.005	0.182	3	0.972	0.082
	2	3	1.040	0.116	3	1.409[b]	0.119	3	1.226	0.086	3	1.389[b]	0.144
	4	3	0.931	0.214	3	0.918	0.218	3	0.859	0.033	3	0.882	0.056
	8	3	0.687[a]	0.135	3	0.843	0.152	3	0.644	0.098	3	0.743	0.172
	12	3	0.766	0.135	3	0.868	0.132	3	0.676	0.073	3	0.693	0.127
	16	3	0.541[a]	0.069	3	0.709[b]	0.081	3	0.567	0.104	3	0.555	0.099
	20	3	0.607[a]	0.161	3	0.609	0.156	3	0.501	0.128	3	0.514	0.165
	24	3	0.485[a]	0.072	3	0.548	0.033	3	0.469	0.074	3	0.364	0.002
	48	3	0.414[a]	0.264	3	0.395	0.129	3	0.345	0.031	3	0.402	0.019
	72	3	0.487[a]	0.310	3	0.464	0.152	3	0.406	0.037	3	0.473	0.022

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-13. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	30.2	4.5	3	34.6	6.5	3	30.3	3.3	3	34.0	1.3
	2	3	26.4	5.3	3	33.2	3.1	3	41.9	15.4	3	36.9	6.9
	4	3	42.8	3.2	3	29.9	0.8	3	34.4	4.6	3	42.6	5.0
	8	3	56.8	1.1	3	51.3	0.7	3	48.1	3.7	3	43.5	2.6
	12	3	61.5	5.2	3	49.1	4.6	3	40.3	2.8	3	40.5	5.1
	16	3	69.0	3.8	3	32.9	6.8	3	51.4	6.4	3	47.8	1.2
	20	3	91.1	11.2	3	78.4	10.6	3	64.3	9.8	3	65.8	1.9
	24	3	124.7	8.5	3	93.4	5.0	3	85.4	14.6	3	91.9	24.9
	48	2	138.7	6.9	3	126.0	7.5	3	122.9	14.8	3	103.2	14.0
	72	3	214.1	68.5	3	203.0	45.9	3	183.9	16.8	3	147.2	24.5
171	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	32.8	3.4	3	28.7	6.5	3	29.0	0.6	3	30.8	3.4
	2	3	29.0	9.8	3	30.4	3.1	3	30.4	2.7	3	31.6	3.6
	4	3	39.1	4.3	3	28.0	1.8	3	29.5	2.2	3	34.1	4.6
	8	3	43.5	9.1	3	39.4	2.8	3	34.0	4.8	3	37.2	4.6
	12	3	36.0	8.6	3	34.5	4.1	3	25.3	1.9	3	29.9	3.2
	16	3	34.4	4.1	3	25.1	6.3	3	28.0	2.1	3	29.9	0.9
	20	3	30.2	0.6	3	34.0	7.5	3	26.2	1.3	3	34.4	3.4
	24	3	40.0	1.5	3	34.8	7.4	3	30.1	2.6	3	37.7	7.2
	48	3	12.3	3.6	3	15.6	1.0	3	13.7	0.4	3	22.7	2.2
	72	3	13.0	1.2	3	9.2	1.3	3	8.8	2.0	3	13.0	2.7

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

TABLE H-14. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	1.000	0.150	3	1.147	0.215	3	1.006	0.108	3	1.125	0.044
	2	3	1.000	0.199	3	1.254	0.117	3	1.586	0.582	3	1.396	0.260
	4	3	1.000	0.076	3	0.699	0.020	3	0.803	0.108	3	0.996	0.116
	8	3	1.000	0.019	3	0.904	0.013	3	0.847	0.065	3	0.765	0.046
	12	3	1.000	0.084	3	0.799	0.076	3	0.656	0.046	3	0.659	0.084
	16	3	1.000	0.055	3	0.478	0.099	3	0.745	0.093	3	0.694	0.017
	20	3	1.000	0.123	3	0.860	0.117	3	0.706	0.108	3	0.723	0.021
	24	3	1.000	0.068	3	0.749	0.040	3	0.685	0.117	3	0.737	0.200
	48	2	1.000	0.050	3	0.908	0.054	3	0.886	0.107	3	0.744	0.101
	72	3	1.000	0.320	3	0.948	0.215	3	0.859	0.079	3	0.688	0.114
171	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	3	1.088	0.112	3	0.953	0.215	3	0.963	0.019	3	1.020	0.111
	2	3	1.098	0.370	3	1.151	0.118	3	1.150	0.102	3	1.197	0.136
	4	3	0.913	0.100	3	0.655[b]	0.042	3	0.690[b]	0.051	3	0.797	0.107
	8	3	0.766	0.160	3	0.693	0.048	3	0.598	0.085	3	0.655	0.080
	12	3	0.585[a]	0.140	3	0.561	0.067	3	0.411[b]	0.031	3	0.486	0.051
	16	3	0.499[a]	0.059	3	0.364	0.091	3	0.406	0.030	3	0.434	0.013
	20	3	0.331[a]	0.006	3	0.373	0.083	3	0.288	0.014	3	0.378	0.038
	24	3	0.320[a]	0.012	3	0.279	0.059	3	0.241	0.021	3	0.303	0.058
	48	3	0.089[a]	0.026	3	0.112	0.007	3	0.099	0.003	3	0.164[b]	0.016
	72	3	0.061[a]	0.006	3	0.043	0.006	3	0.041[b]	0.009	3	0.061	0.013

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.

[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE H-15. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Picomoles Per Sample) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	58.7	1.4	3	54.6	0.5	3	53.9	6.1	3	60.0	1.5
	1	3	54.5	4.2	3	52.9	2.9	3	57.9	11.9	3	56.4	6.8
	2	3	39.5	4.9	3	41.5	3.5	3	37.2	9.3	3	40.1	6.9
	4	3	45.9	1.1	3	41.0	12.9	3	45.4	4.7	3	39.5	1.4
	8	3	58.5	6.4	3	59.2	7.6	3	51.0	4.0	3	45.1	4.3
	12	3	81.1	10.1	3	79.9	10.7	3	69.2	1.4	3	64.1	3.4
	16	3	100.5	5.1	3	110.4	9.0	1	73.9	-	3	72.0	14.7
	20	3	73.4	11.3	3	77.9	13.3	3	68.8	2.9	3	57.0	2.4
	24	3	71.7	3.4	3	71.0	11.9	3	74.5	10.7	3	61.9	2.6
	48	3	88.9	7.3	3	89.2	6.1	3	84.6	3.9	3	68.1	9.7
	72	3	111.8	19.7	3	120.3	0.8	3	101.3	4.1	3	77.9	5.4
171	0	3	65.3	1.8	3	52.0	16.9	3	60.3	5.8	3	62.4	4.3
	1	3	52.0	11.5	3	50.6	3.2	3	48.4	10.7	3	50.6	6.3
	2	3	38.2	6.1	3	42.8	4.5	3	36.3	4.9	3	35.0	5.2
	4	3	43.8	10.7	3	39.5	3.2	3	33.3	2.1	3	38.4	2.0
	8	3	29.8	3.1	3	35.6	11.1	3	29.9	5.9	3	31.2	5.2
	12	3	43.1	11.2	3	36.4	4.1	3	33.6	2.1	3	40.7	5.6
	16	3	33.9	5.2	3	41.0	15.7	3	30.1	6.5	3	30.3	7.9
	20	3	37.2	9.3	3	34.0	2.4	3	28.3	4.7	3	29.0	5.0
	24	3	22.5	5.9	3	22.9	2.1	3	19.2	3.5	3	25.2	1.0
	48	3	14.6	3.1	3	19.3	3.4	3	14.9	2.5	3	21.8	5.2
	72	3	8.5	1.2	3	7.9	2.1	3	6.4	1.3	3	9.0	1.0

TABLE H-16. DESCRIPTIVE STATISTICS FOR NAD^+ CONCENTRATIONS (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.024	3	0.930	0.009	3	0.919	0.103	3	1.022	0.026
	1	3	1.000	0.077	3	0.970	0.053	3	1.062	0.218	3	1.034	0.124
	2	3	1.000	0.123	3	1.051	0.089	3	0.942	0.235	3	1.015	0.176
	4	3	1.000	0.024	3	0.894	0.281	3	0.990	0.103	3	0.860	0.030
	8	3	1.000	0.109	3	1.011	0.130	3	0.872	0.069	3	0.771	0.073
	12	3	1.000	0.124	3	0.985	0.132	3	0.853	0.017	3	0.790	0.042
	16	3	1.000	0.051	3	1.099	0.089	1	0.736	-	3	0.717	0.147
	20	3	1.000	0.153	3	1.061	0.182	3	0.936	0.039	3	0.777	0.033
	24	3	1.000	0.047	3	0.991	0.166	3	1.039	0.149	3	0.864	0.037
	48	3	1.000	0.082	3	1.004	0.069	3	0.952	0.044	3	0.766	0.109
	72	3	1.000	0.176	3	1.076	0.007	3	0.906	0.037	3	0.697	0.049
171	0	3	1.113	0.030	3	0.886	0.288	3	1.027	0.100	3	1.062	0.073
	1	3	0.954	0.210	3	0.929	0.059	3	0.888	0.197	3	0.928	0.115
	2	3	0.968	0.154	3	1.083	0.114	3	0.920	0.124	3	0.885	0.132
	4	3	0.955	0.234	3	0.862	0.069	3	0.726[b]	0.045	3	0.836	0.043
	8	3	0.509[a]	0.052	3	0.609	0.189	3	0.511	0.102	3	0.534	0.089
	12	3	0.531[a]	0.138	3	0.448	0.051	3	0.414	0.026	3	0.502	0.069
	16	3	0.338[a]	0.051	3	0.408	0.156	3	0.300	0.064	3	0.302	0.078
	20	3	0.507[a]	0.127	3	0.463	0.032	3	0.386[b]	0.063	3	0.395	0.068
	24	3	0.314[a]	0.082	3	0.319	0.030	3	0.268	0.049	3	0.352	0.014
	48	3	0.164[a]	0.035	3	0.217	0.038	3	0.168	0.028	3	0.245[b]	0.059
	72	3	0.076[a]	0.010	3	0.071	0.019	3	0.057	0.012	3	0.080	0.009

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

APPENDIX I

GRAPHS OF CYTOTOXICITY DATA FOR NIACINAMIDE-PRETREATED, HD-EXPOSED CULTURES

FIGURE I-1.

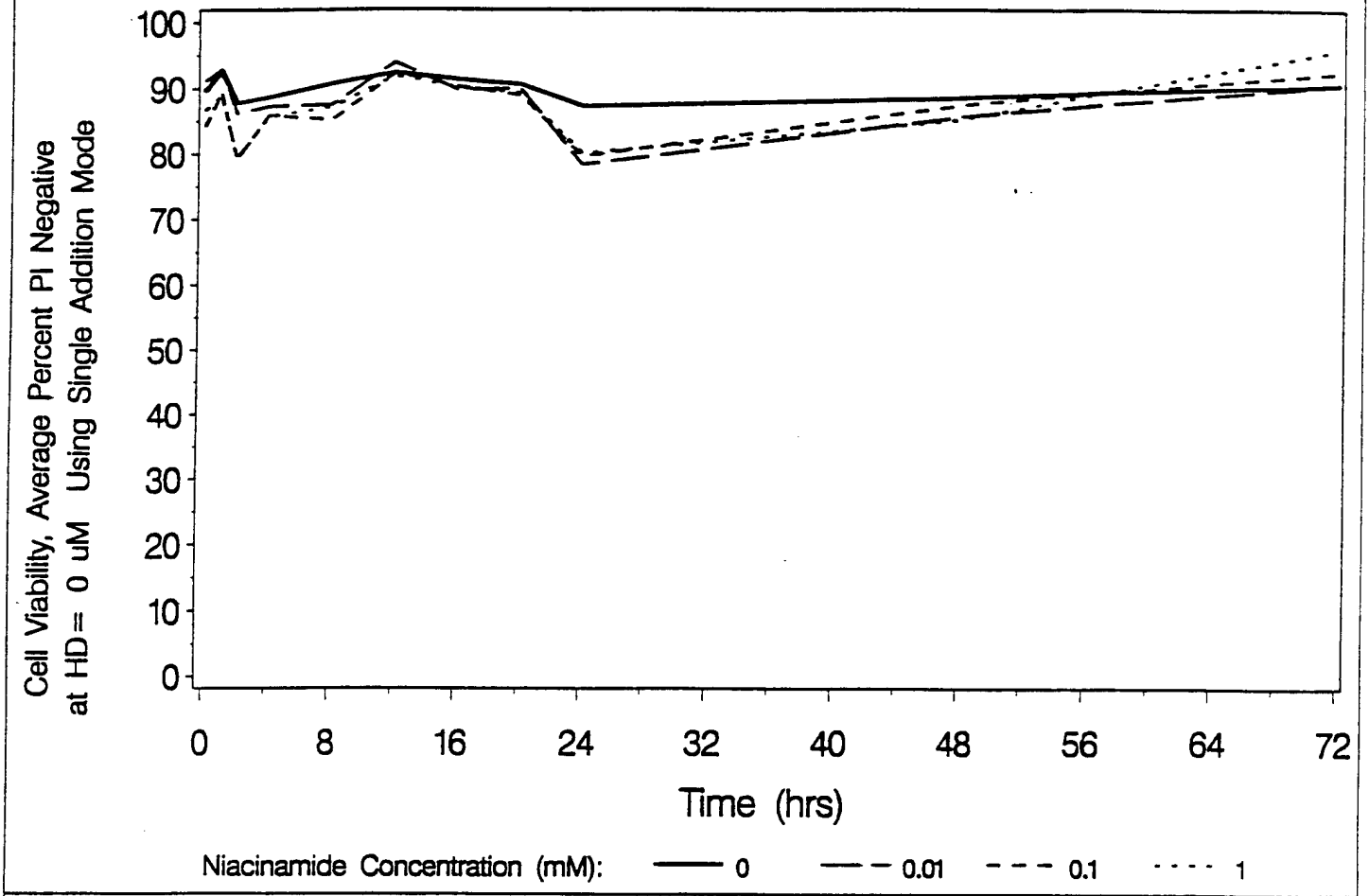


FIGURE I-2.

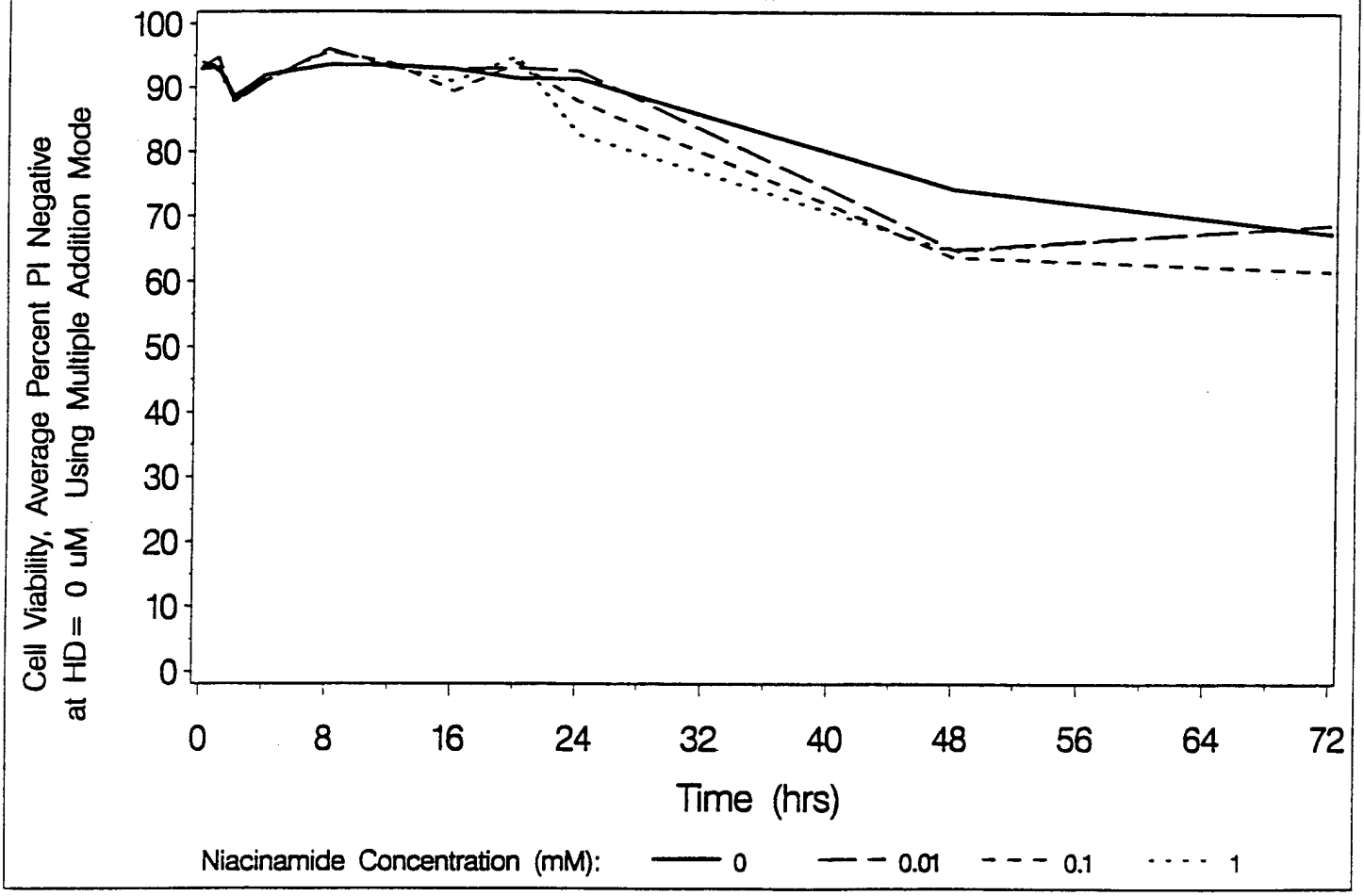


FIGURE I-3.

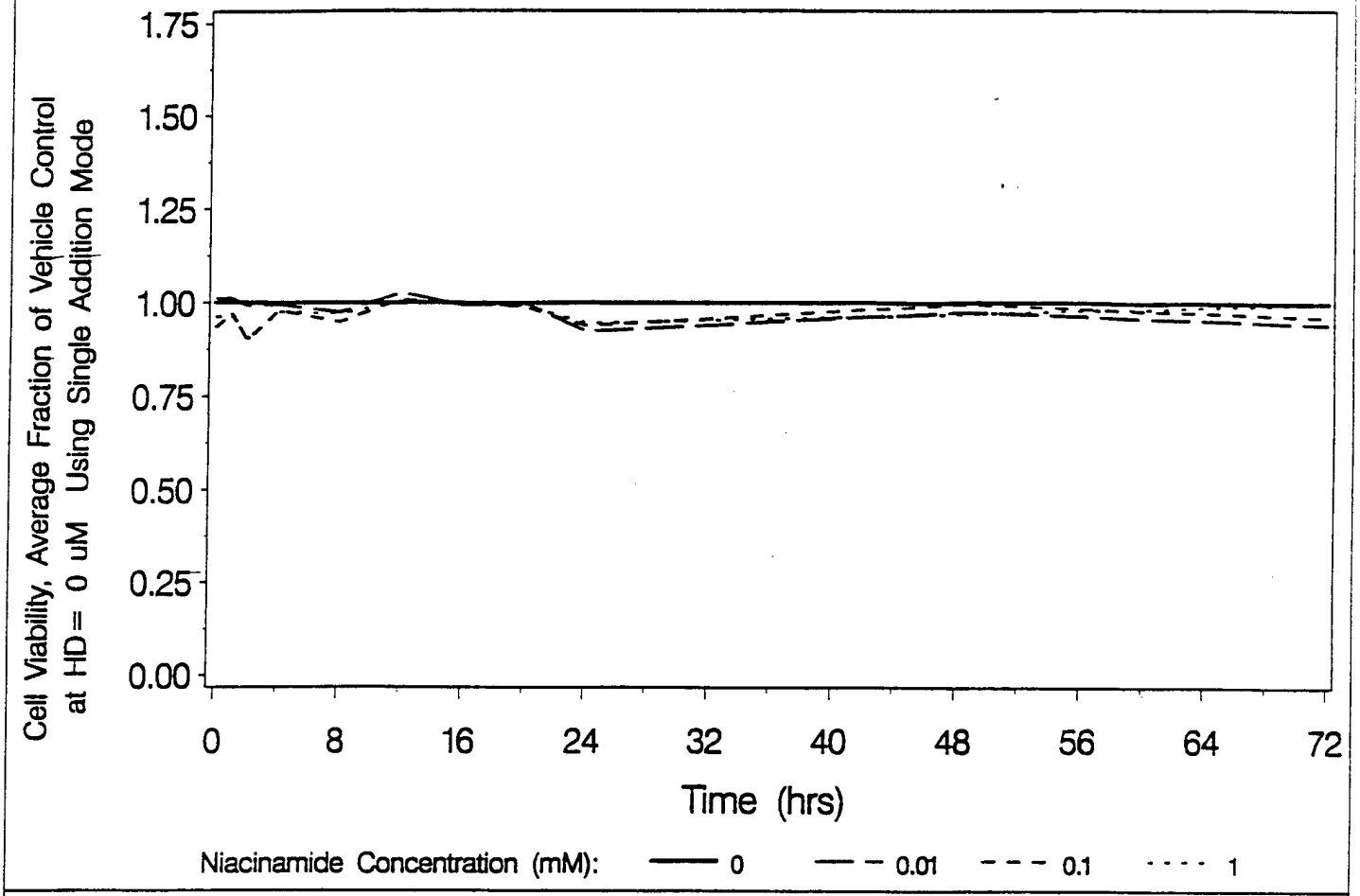


FIGURE 1-4.

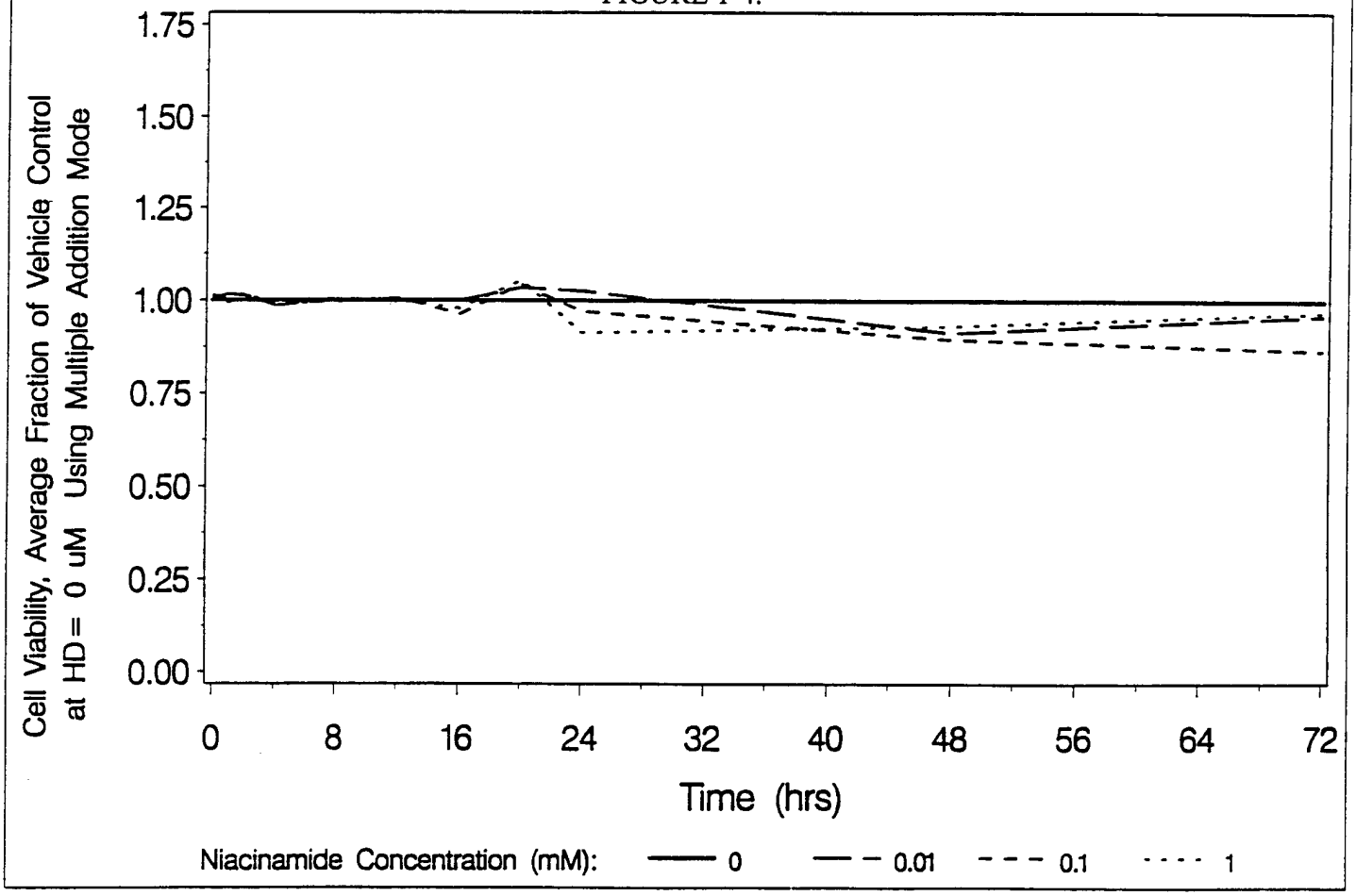


FIGURE I-5.

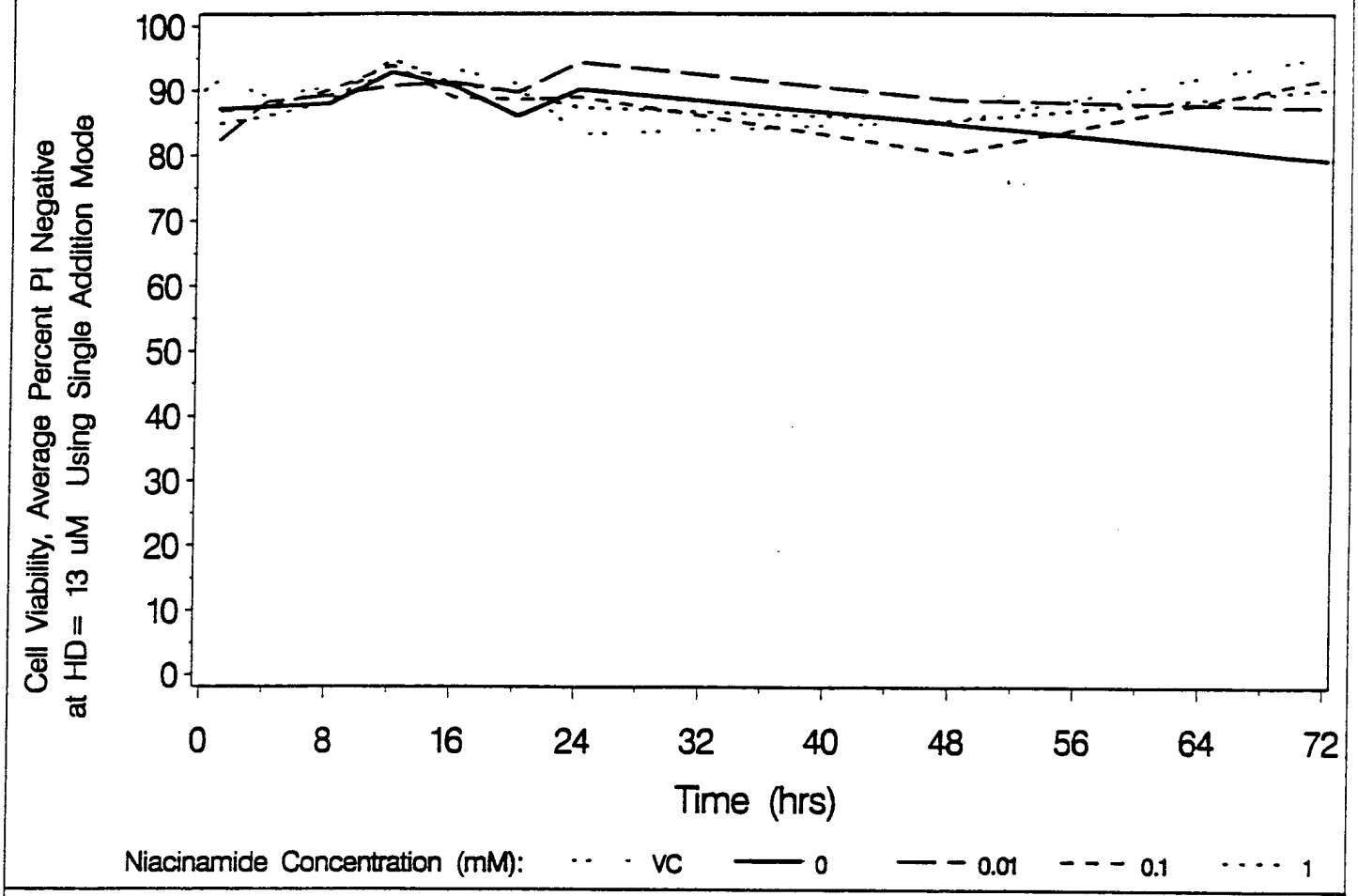


FIGURE I-6.

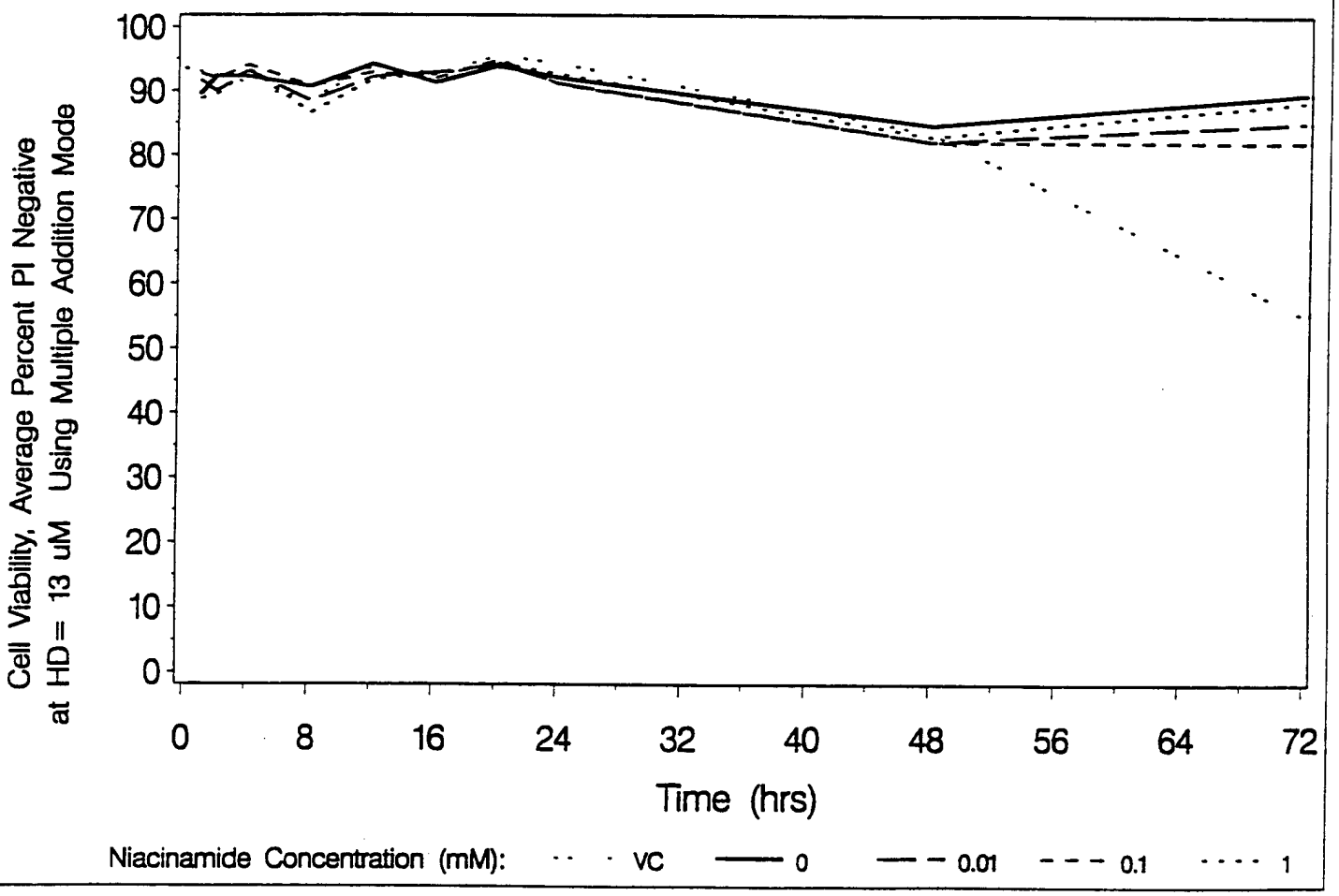


FIGURE I-7.

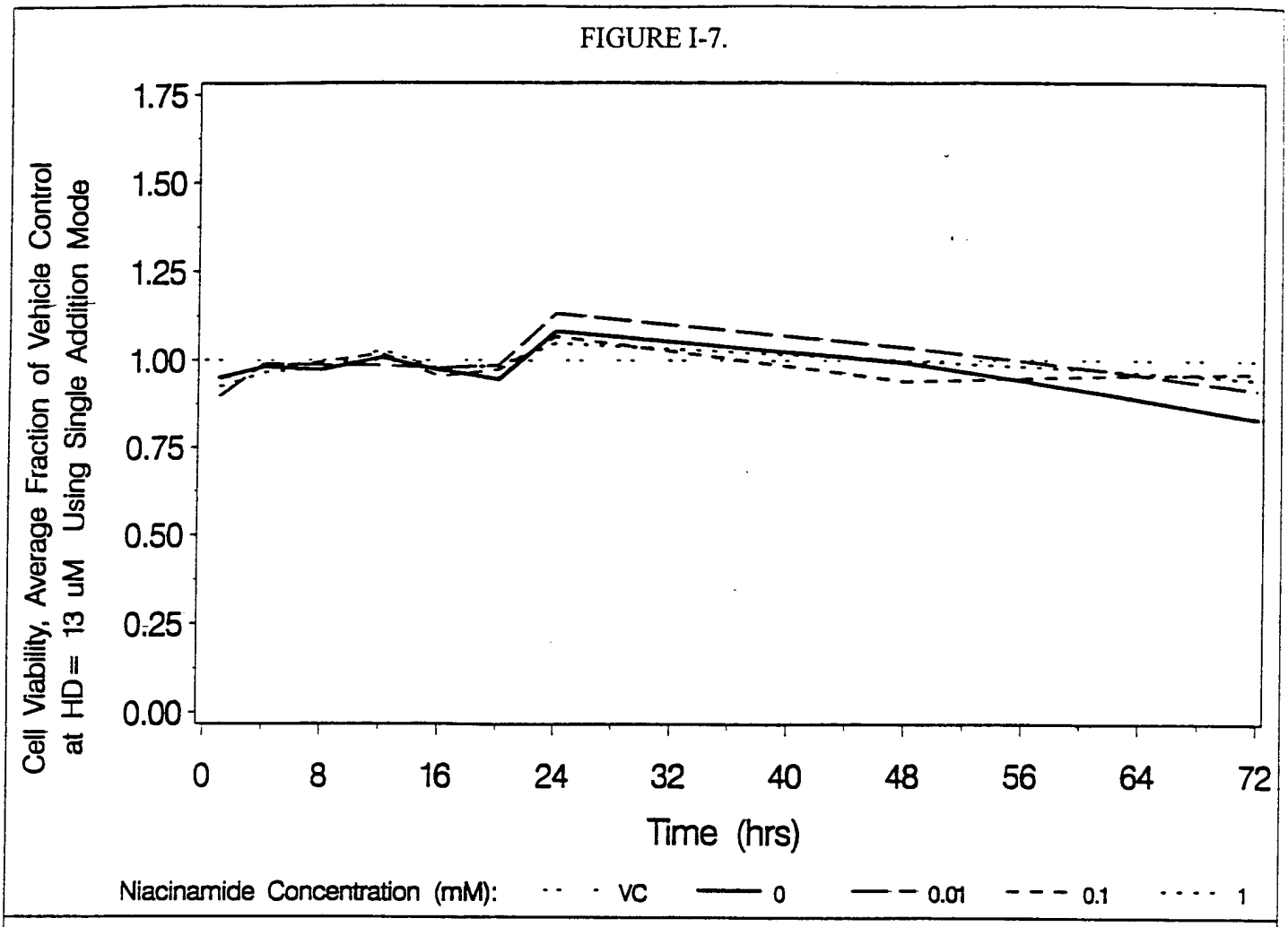


FIGURE I-8.

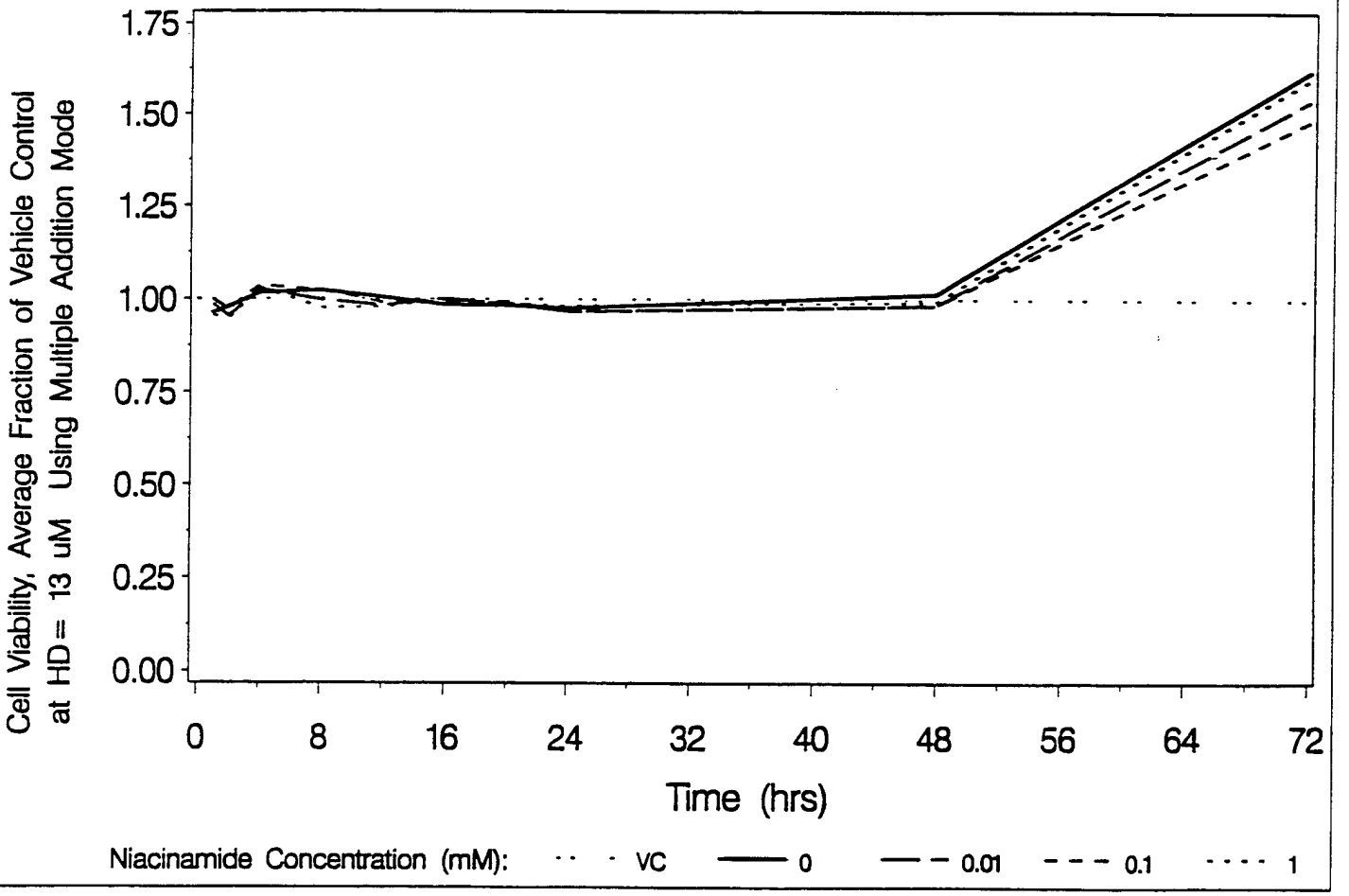


FIGURE I-9.

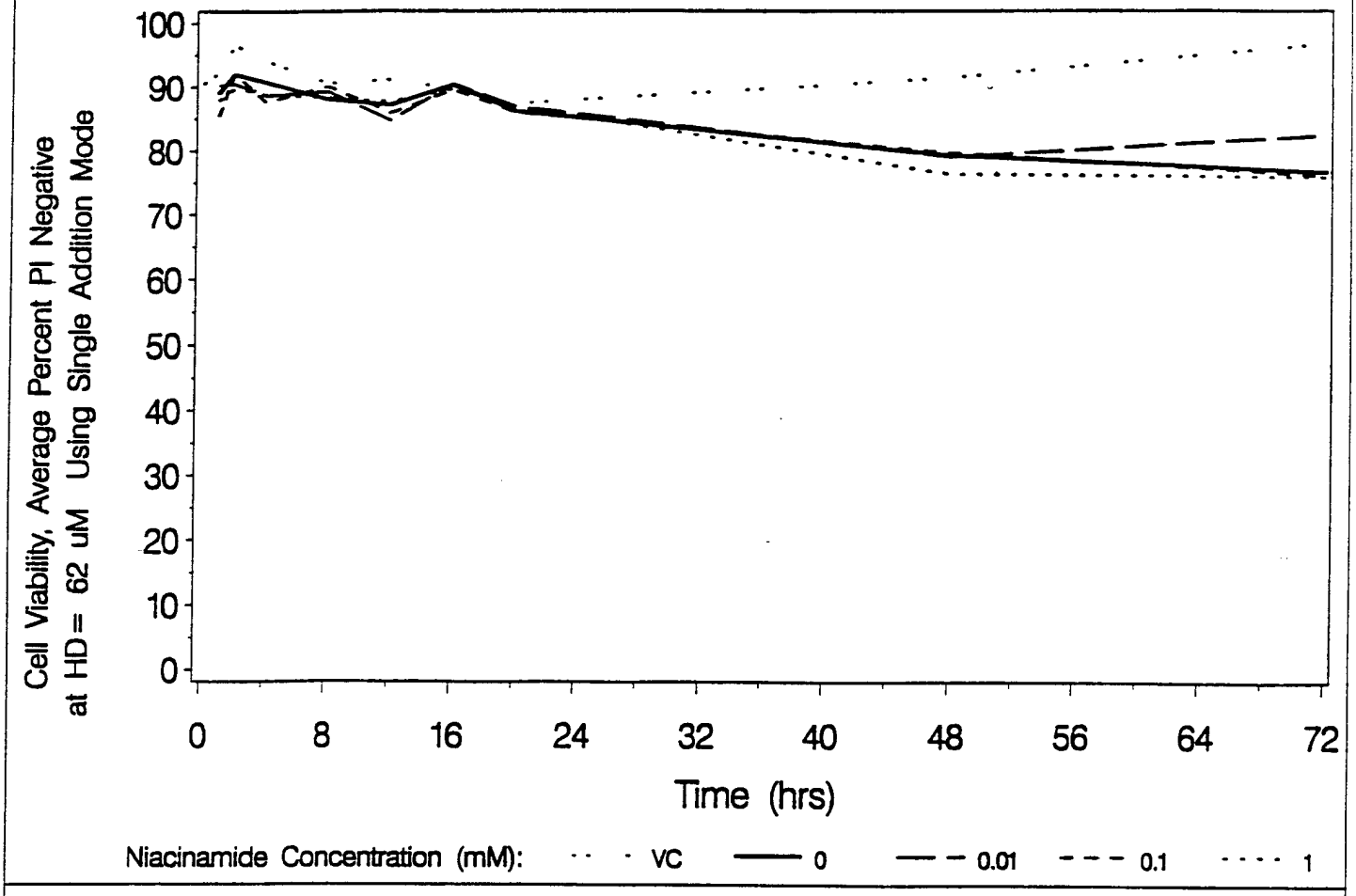


FIGURE I-10.

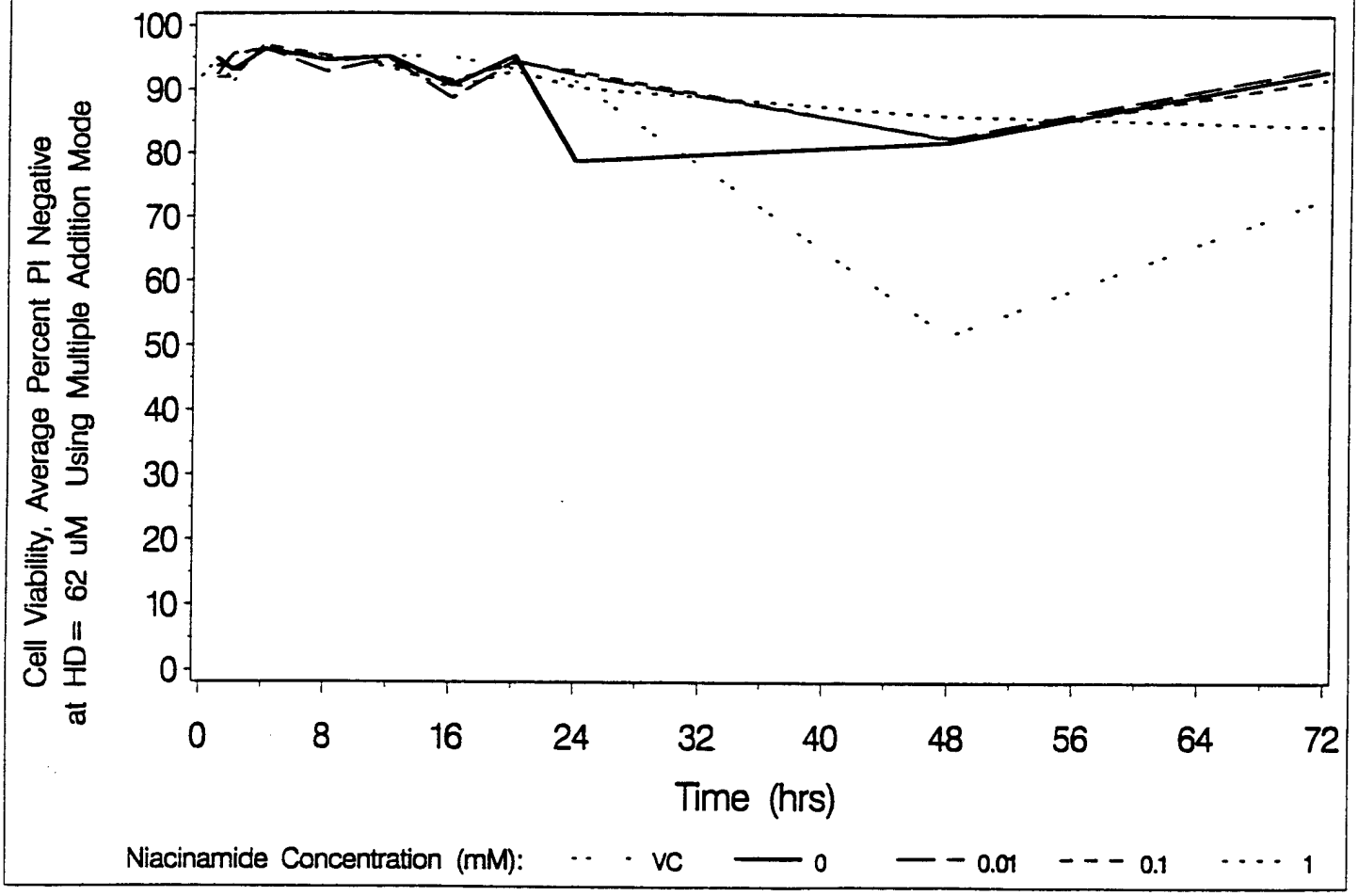


FIGURE I-11.

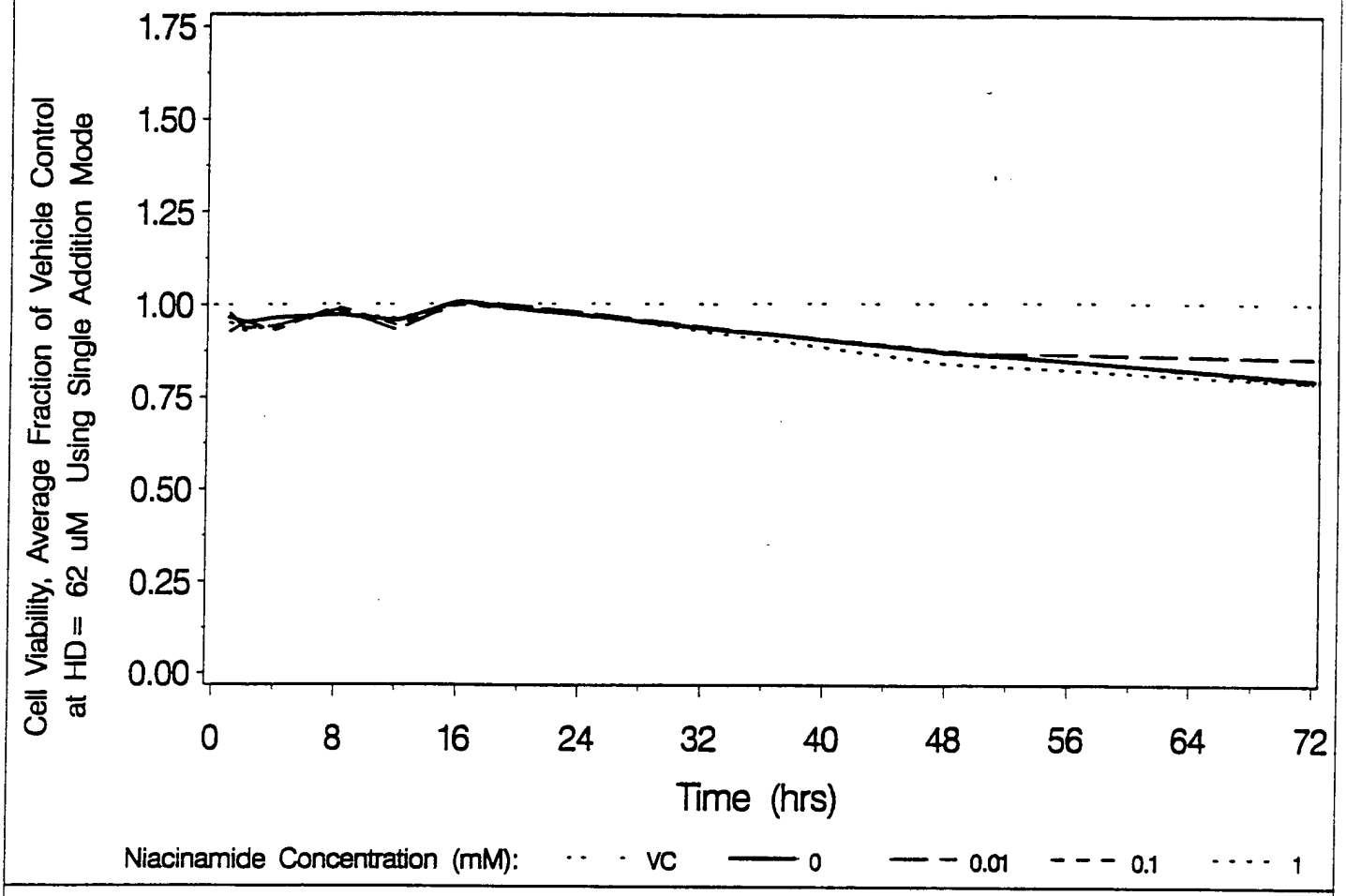


FIGURE I-12.

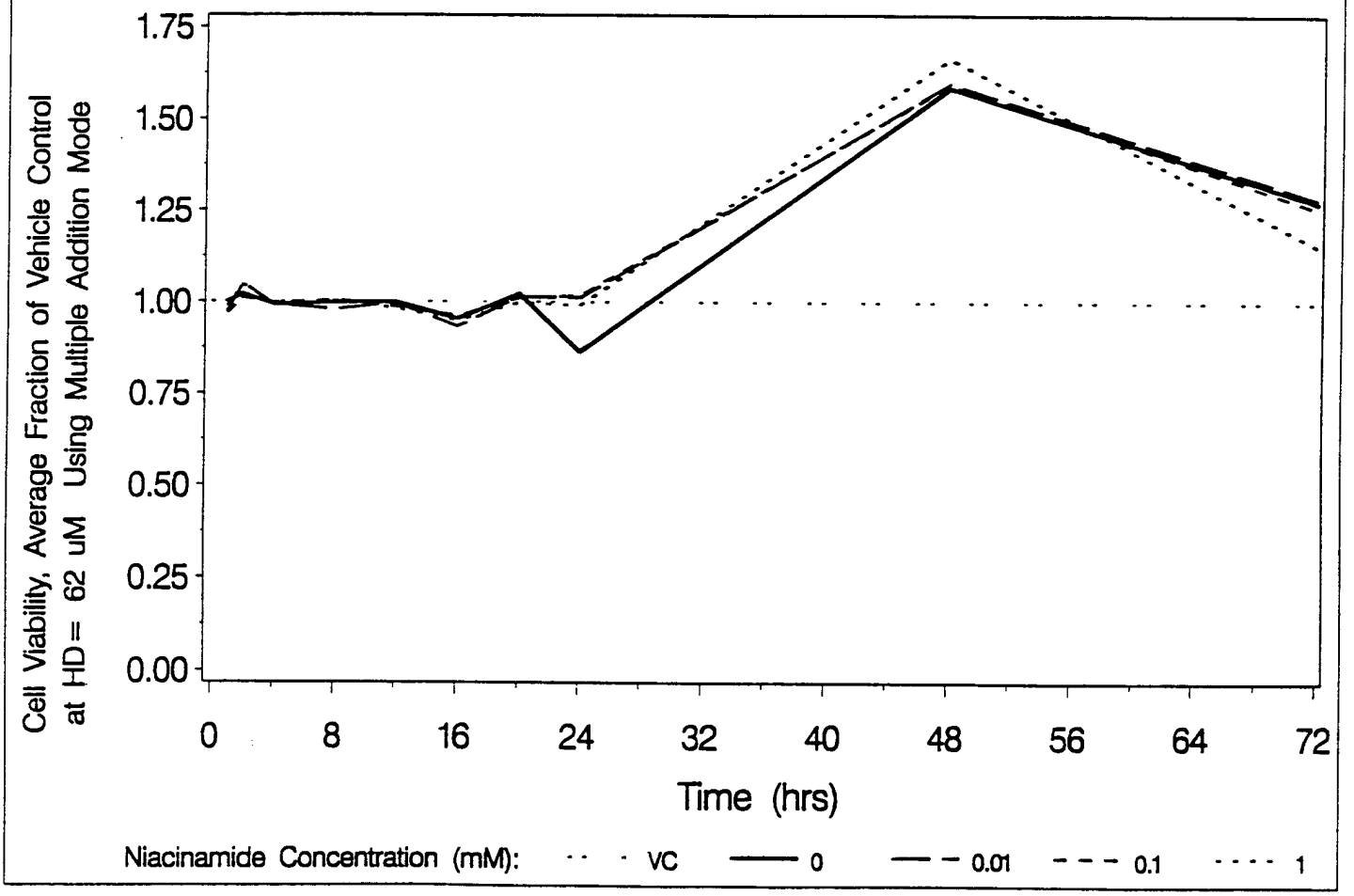


FIGURE I-13.

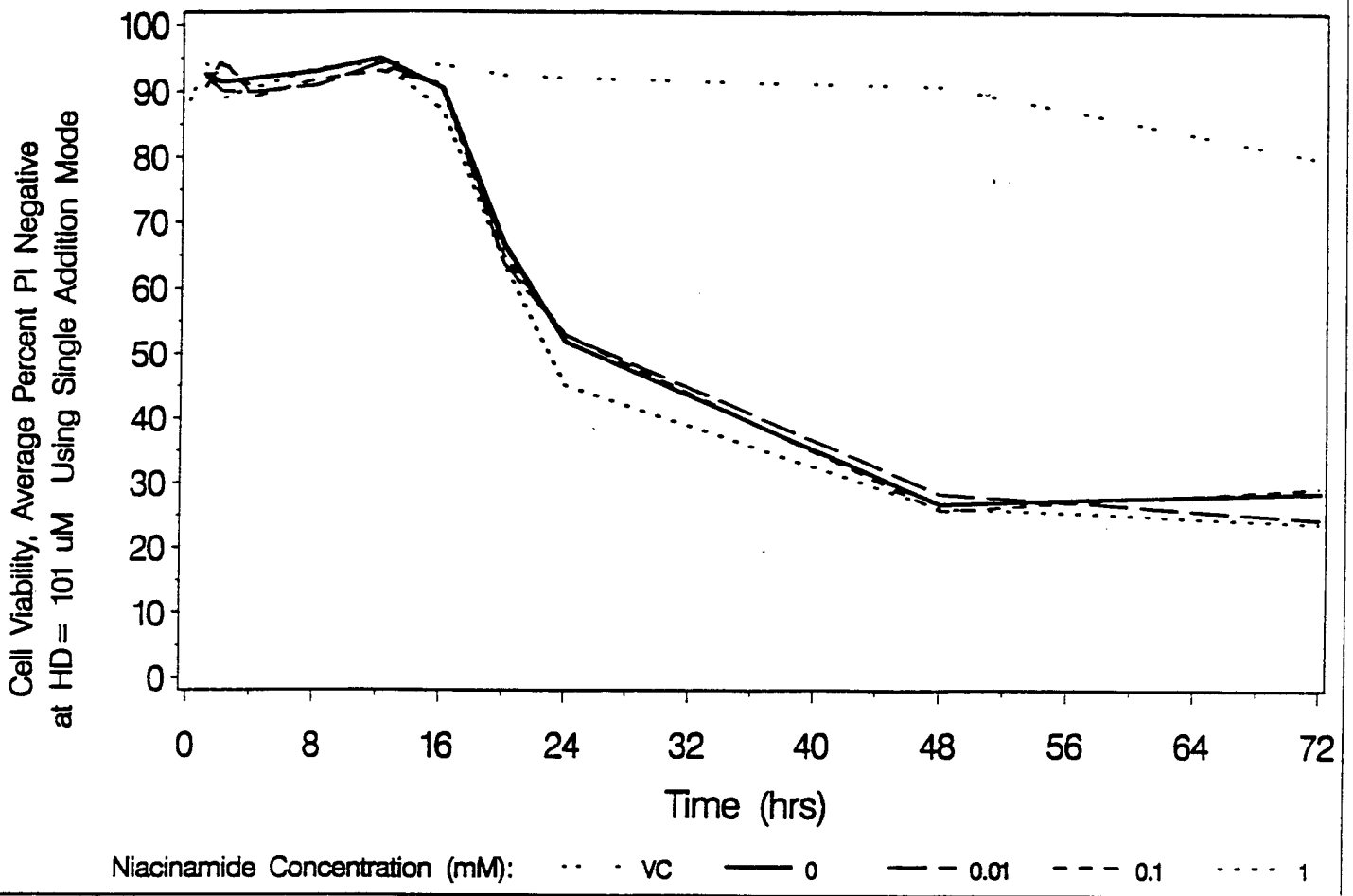


FIGURE I-14.

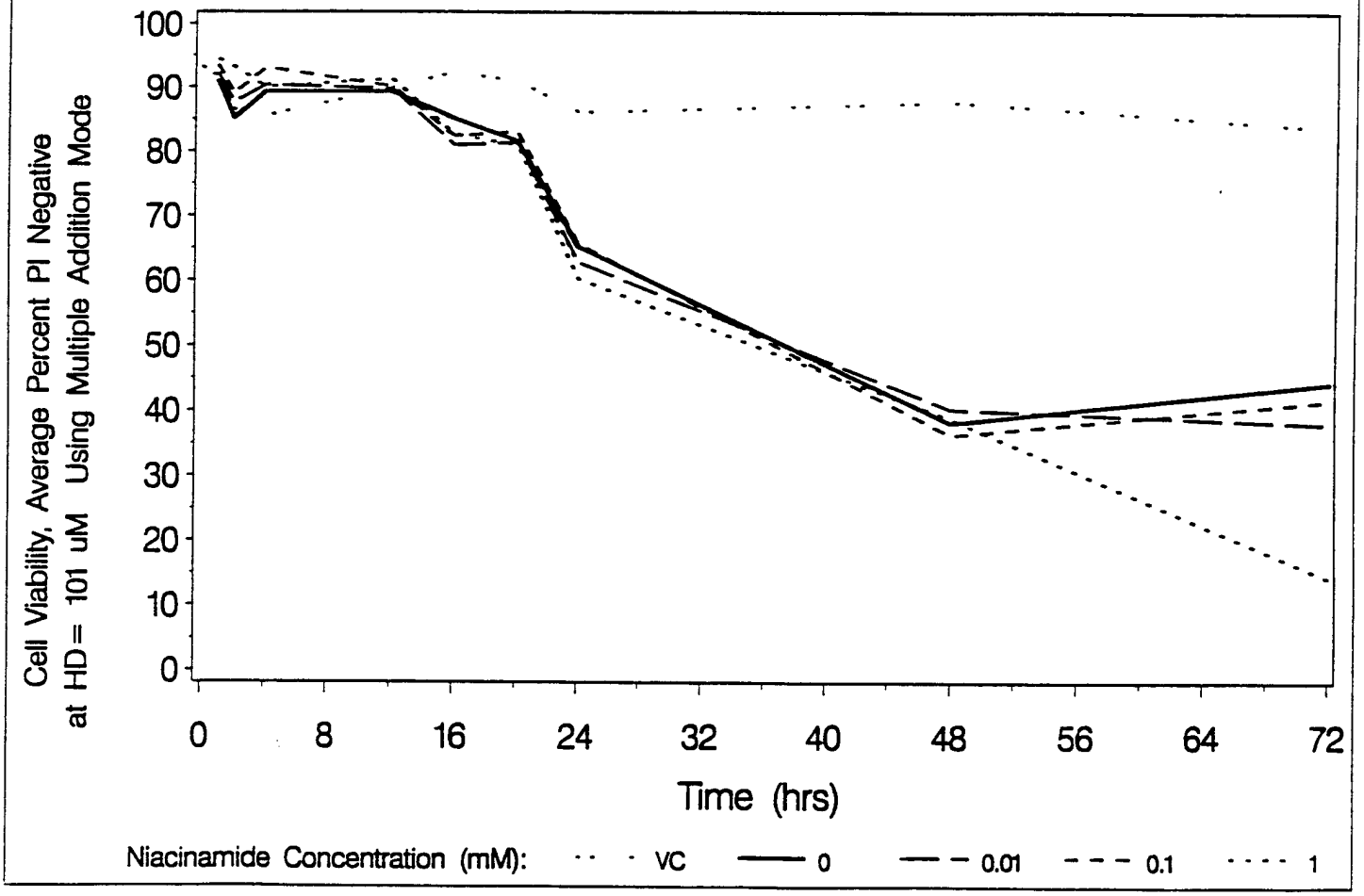


FIGURE I-15.

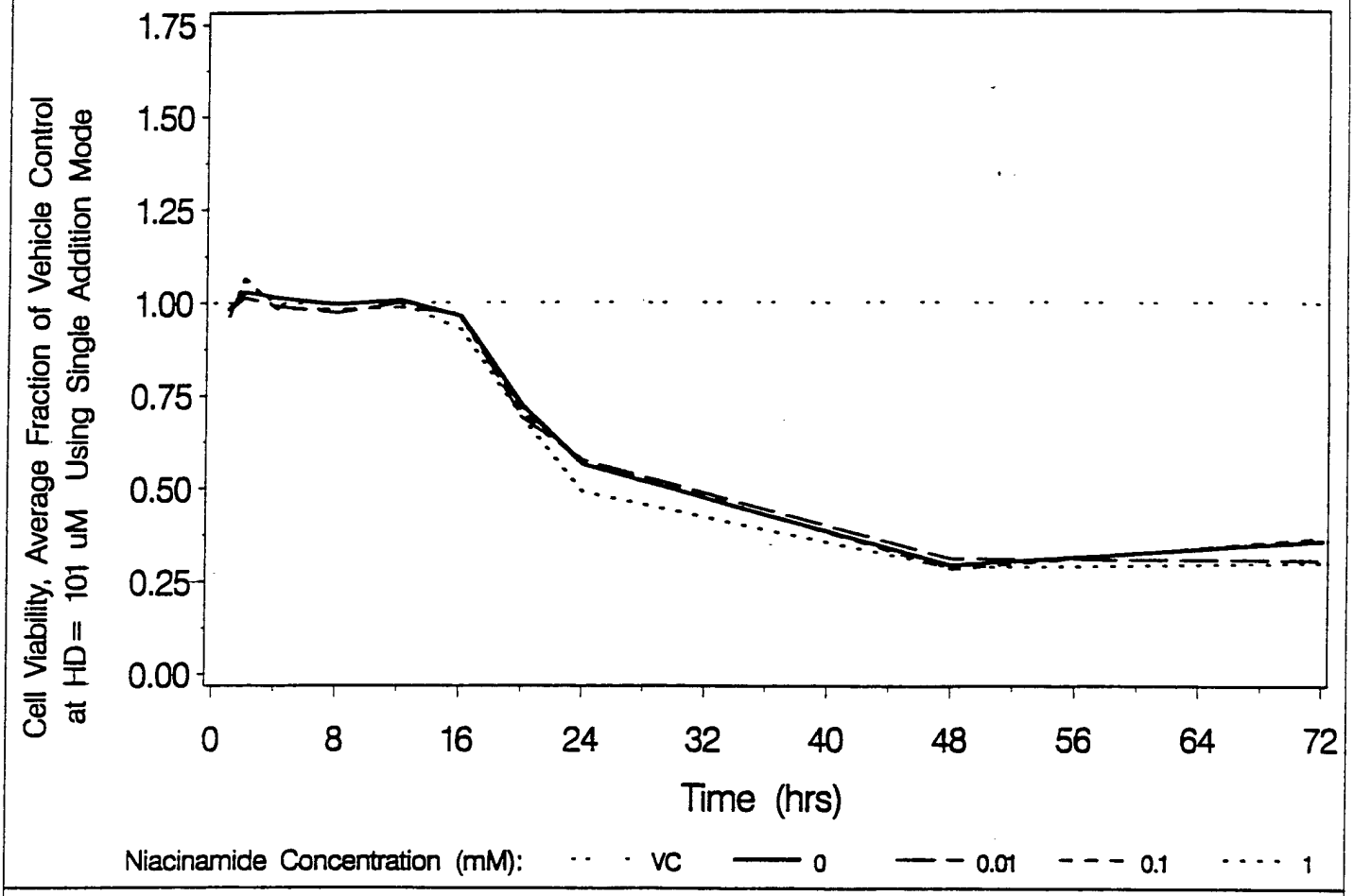


FIGURE I-16.

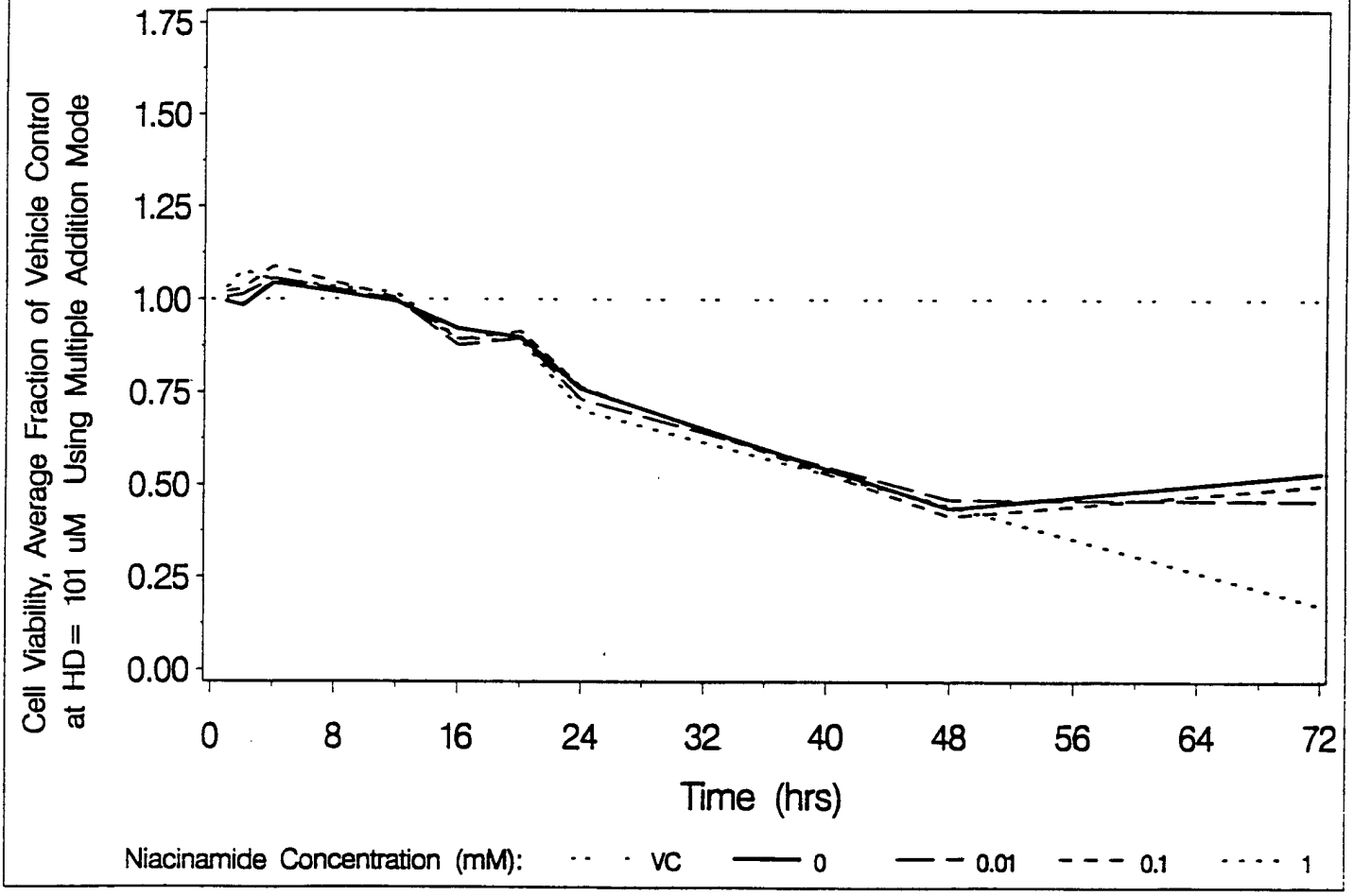


FIGURE I-17.

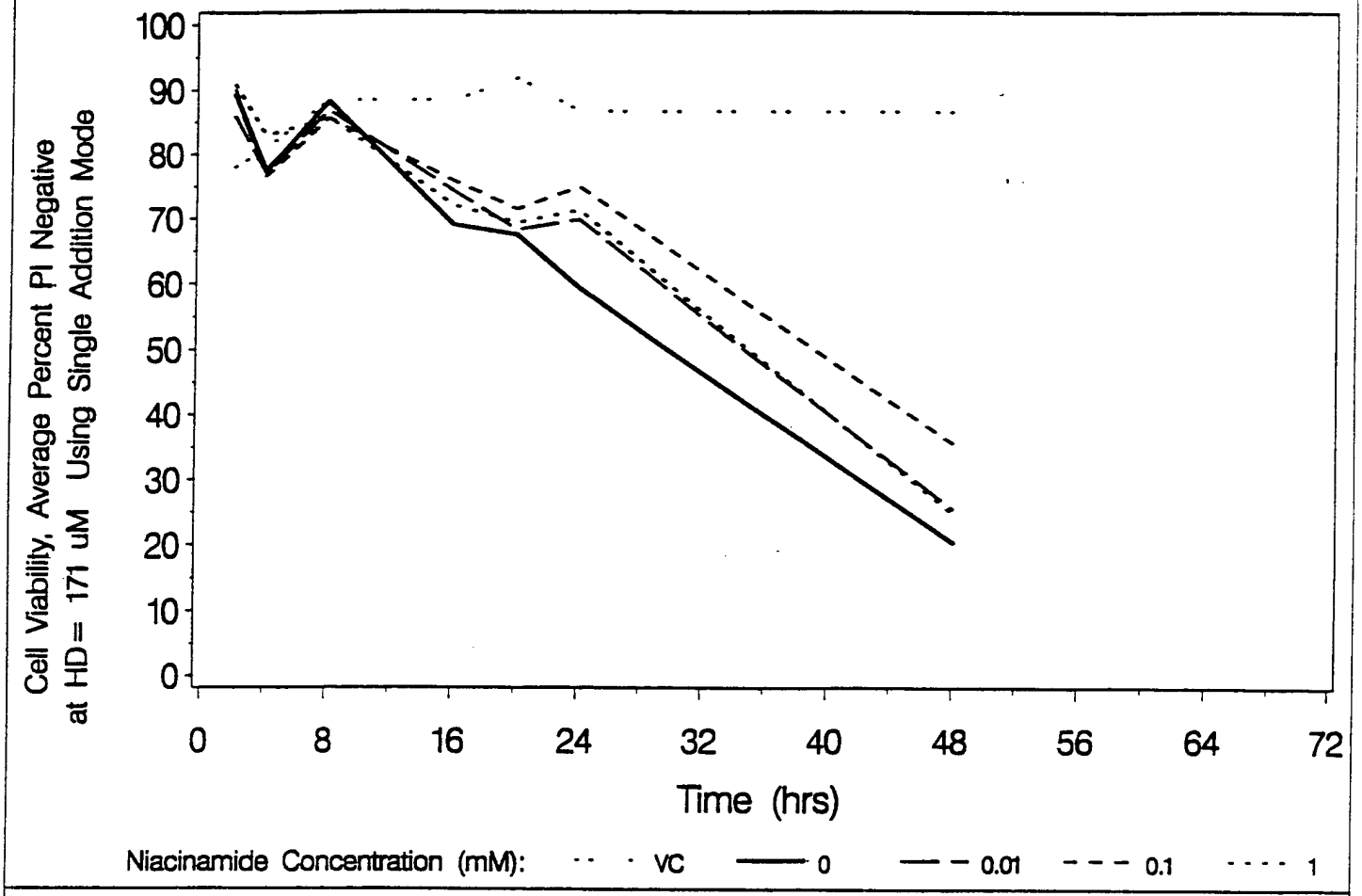


FIGURE I-18.

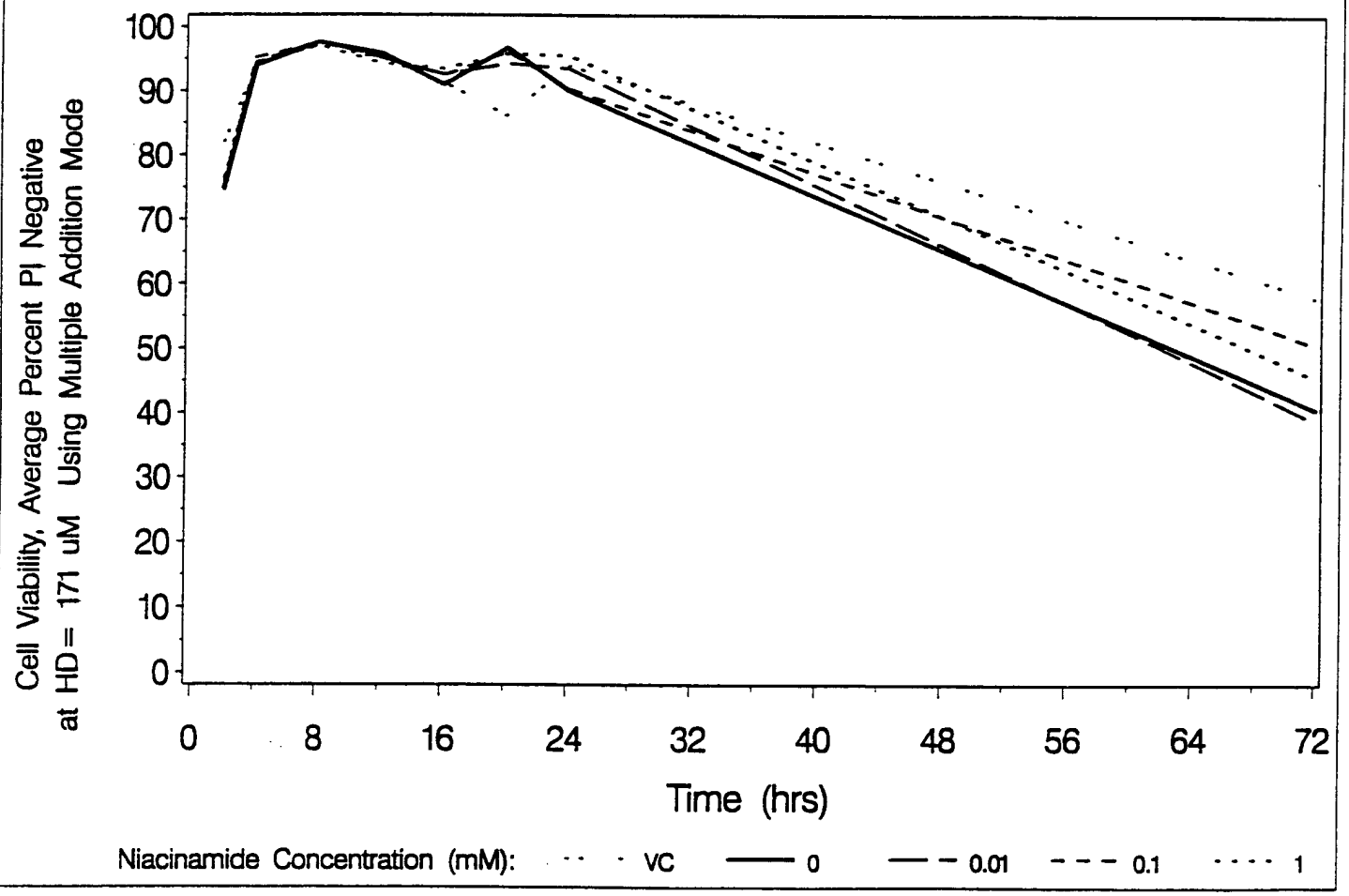


FIGURE I-19.

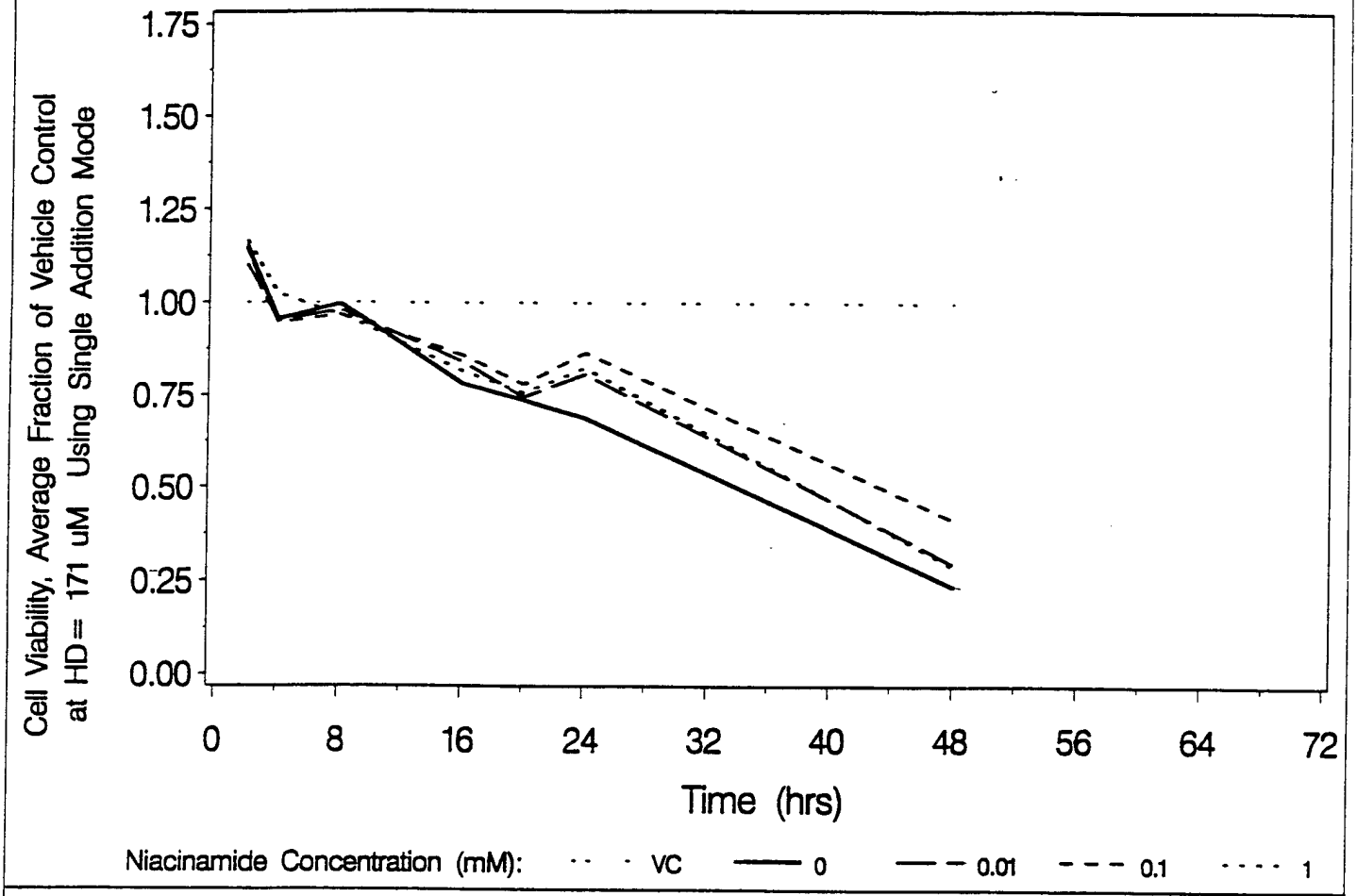
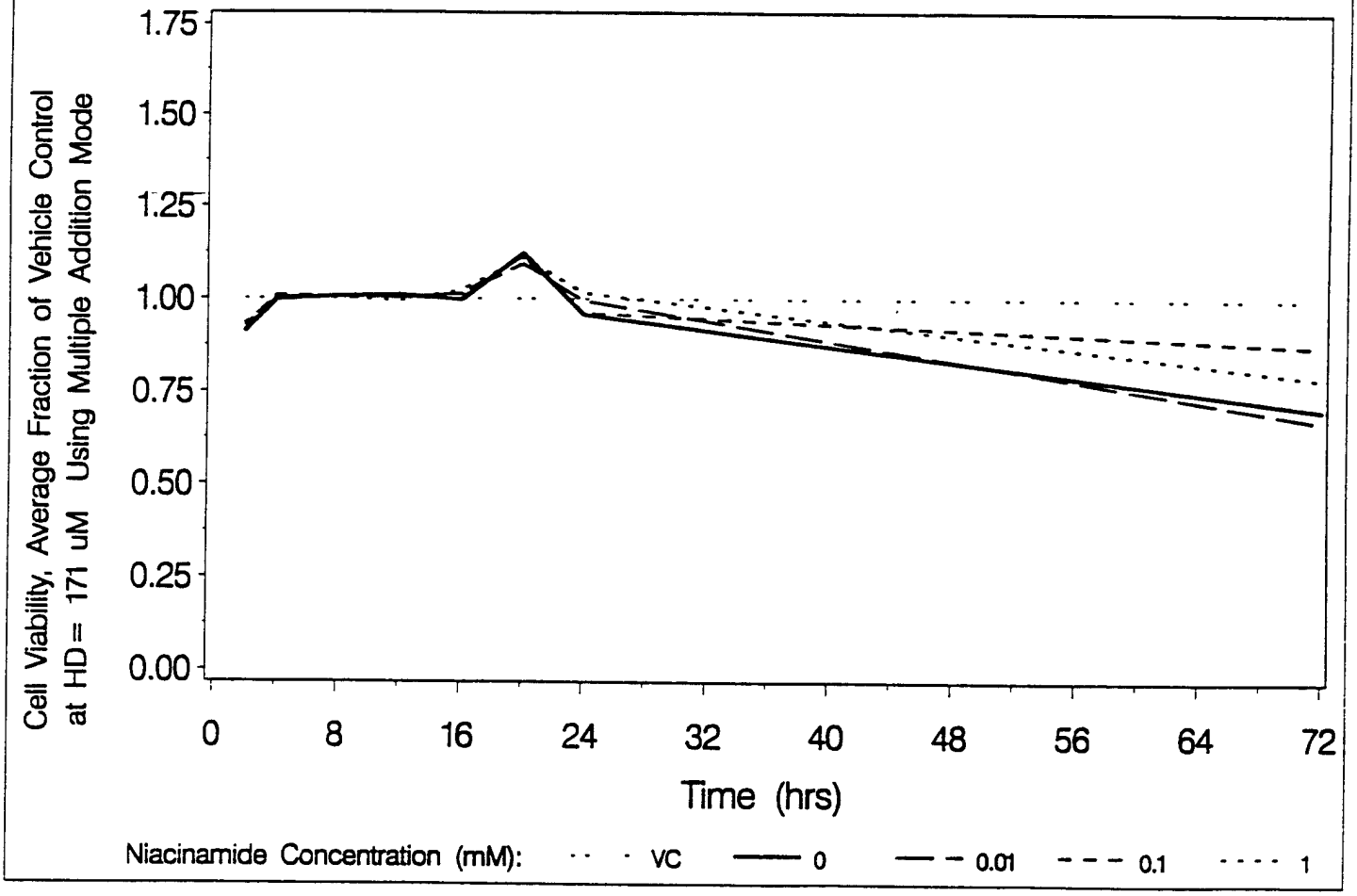


FIGURE I-20.



APPENDIX J

GRAPHS OF CYTOTOXICITY DATA FOR NIACIN-PRETREATED, HD-EXPOSED CULTURES

FIGURE J-1.

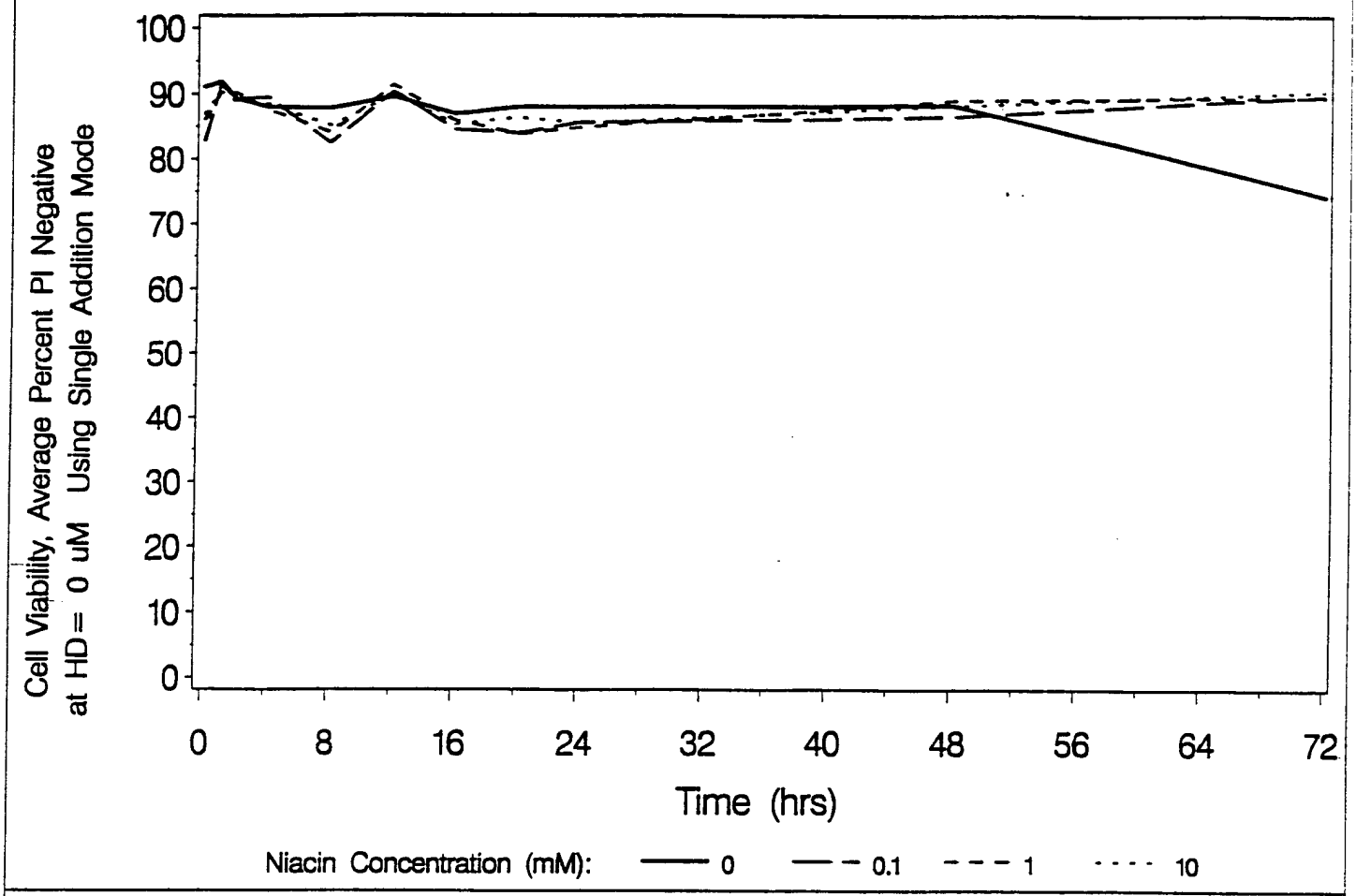


FIGURE J-2.

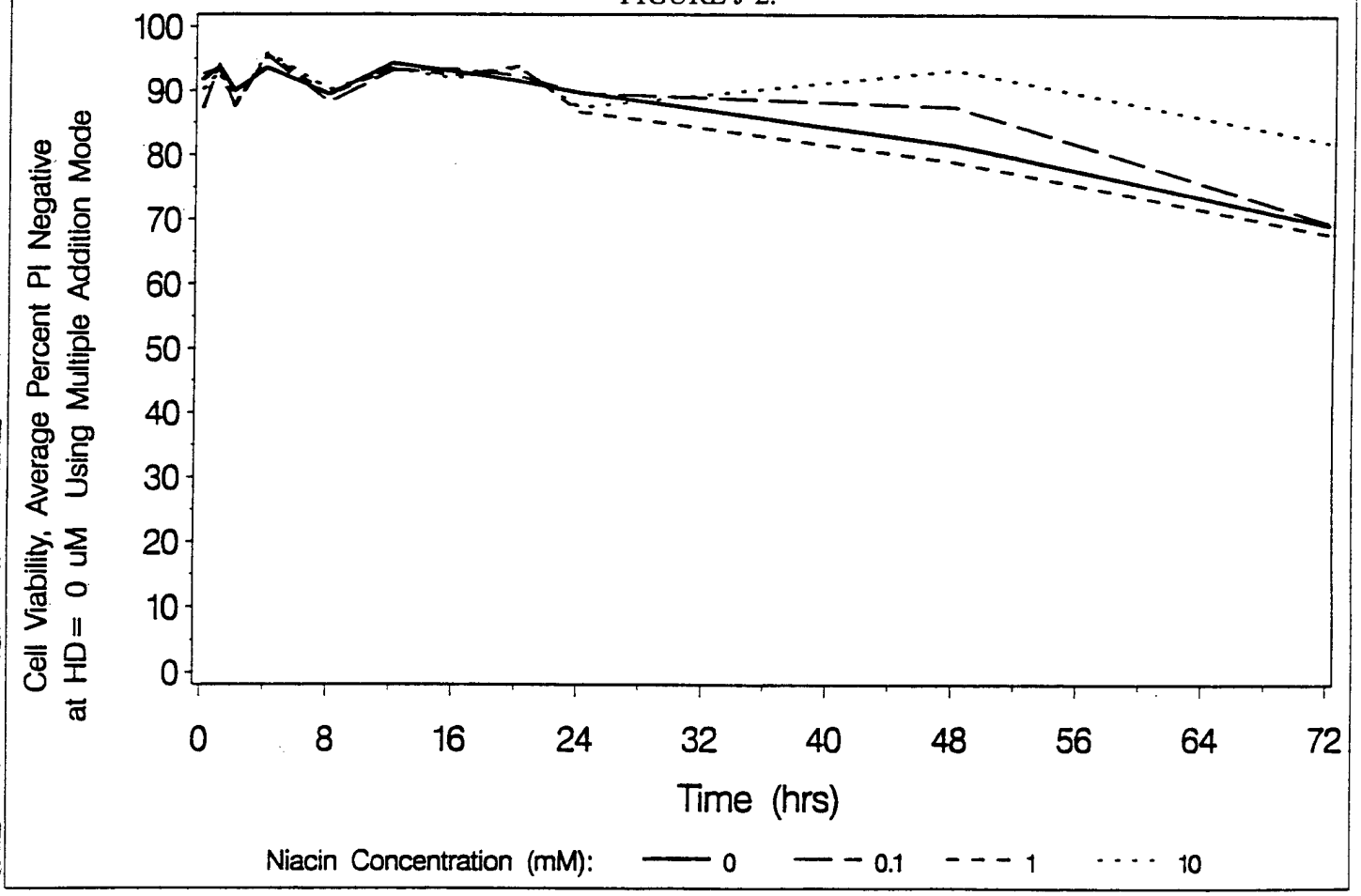


FIGURE J-3.

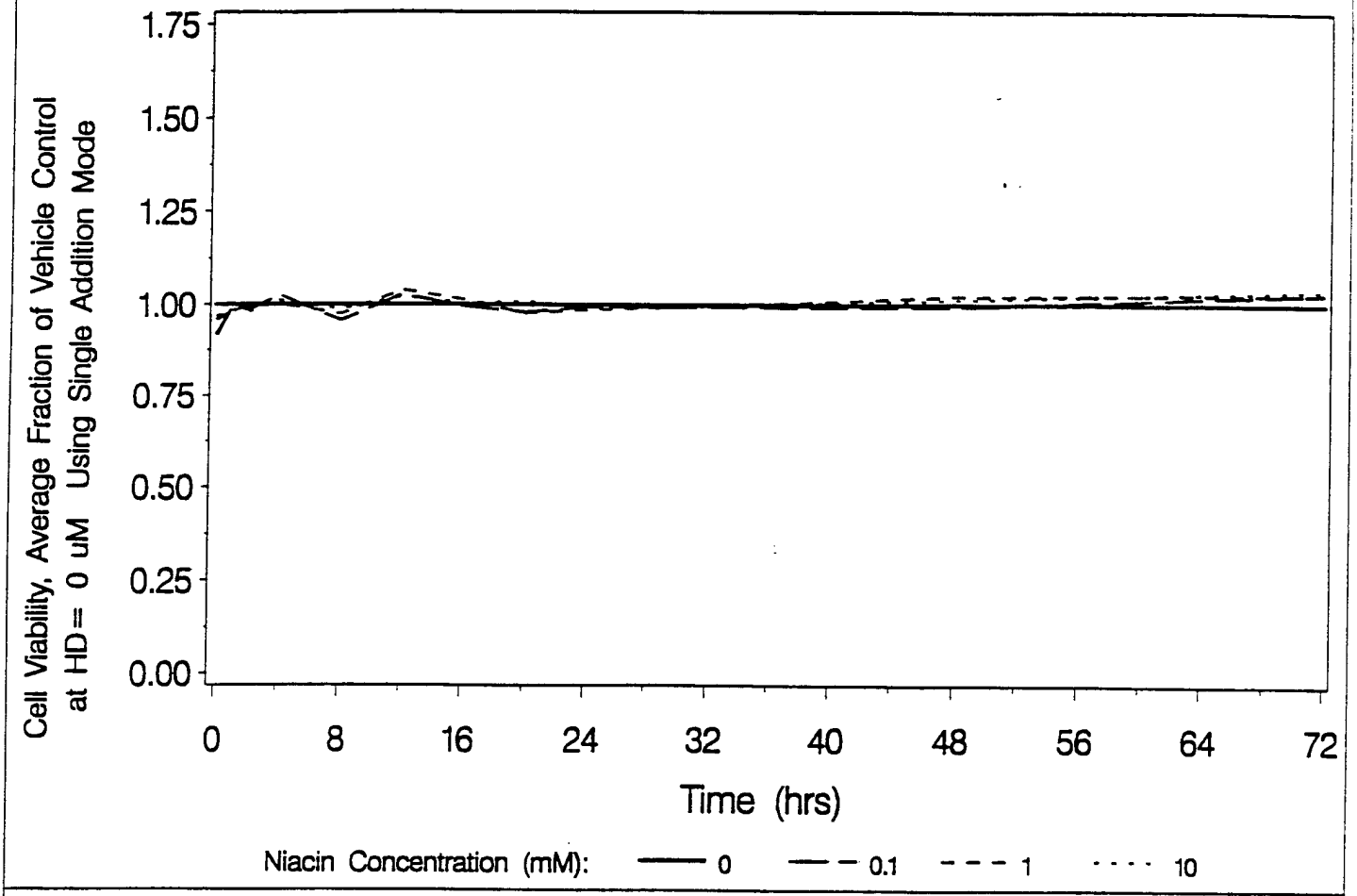


FIGURE J-4.

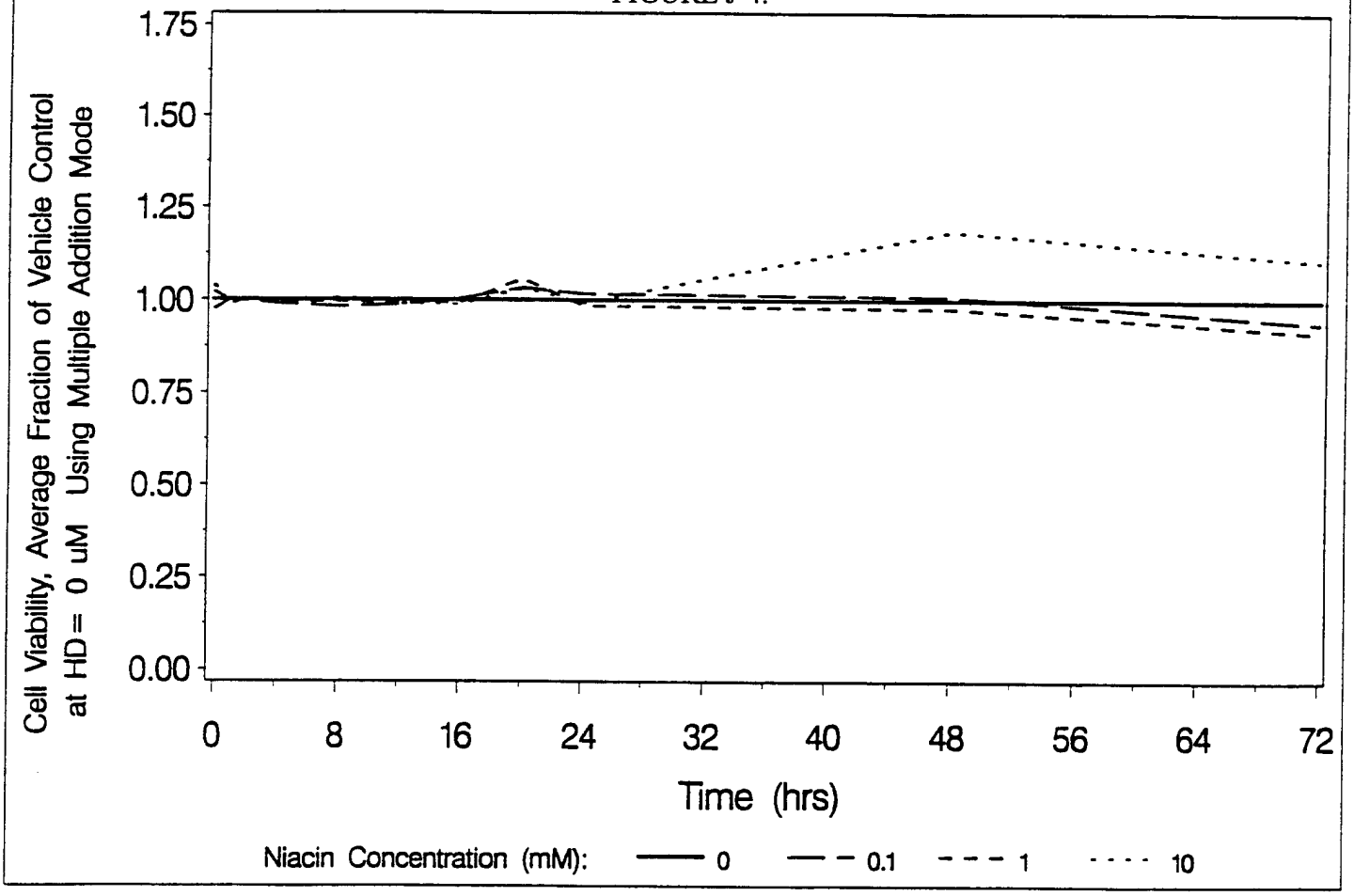


FIGURE J-5.

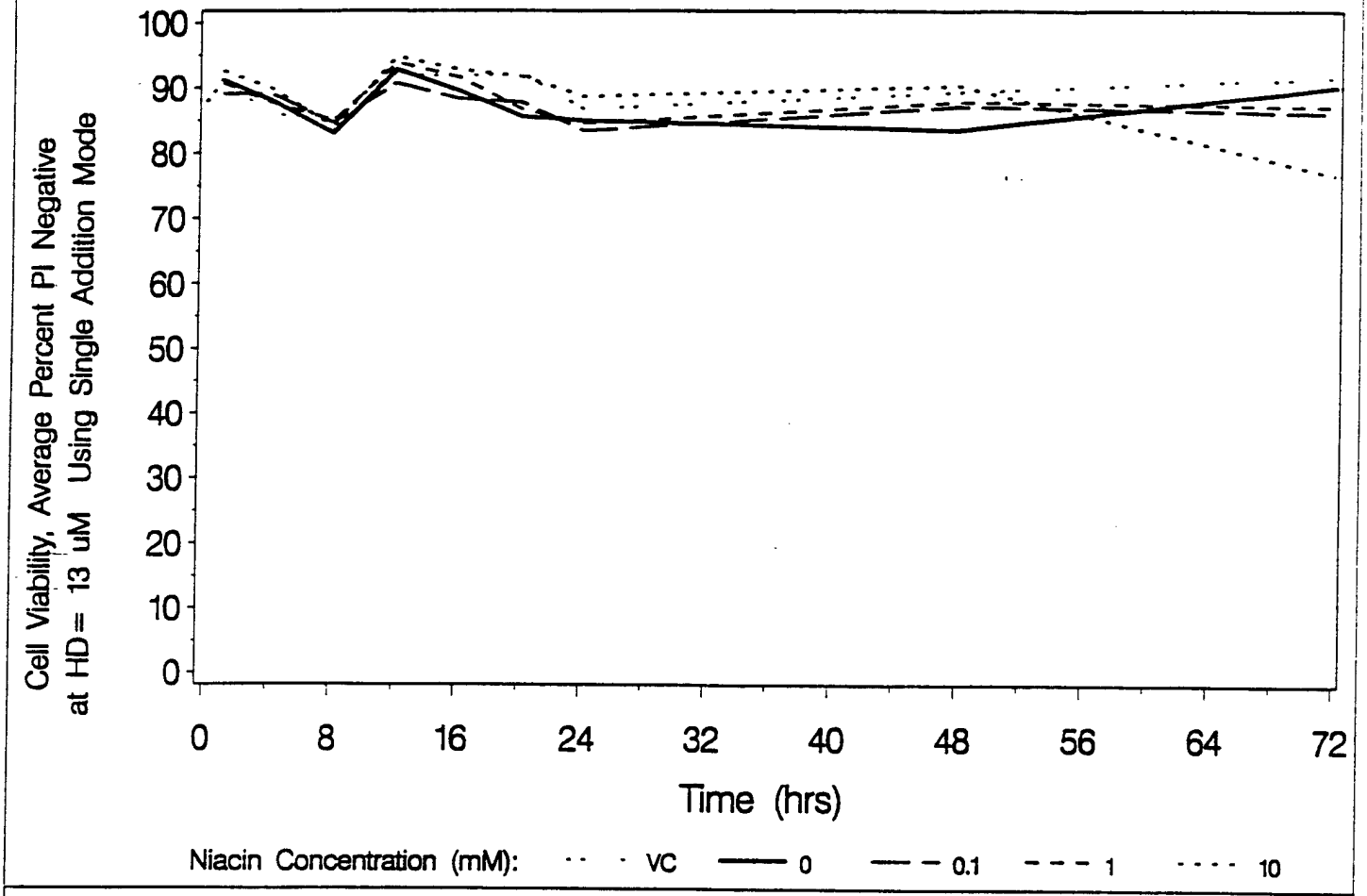


FIGURE J-6.

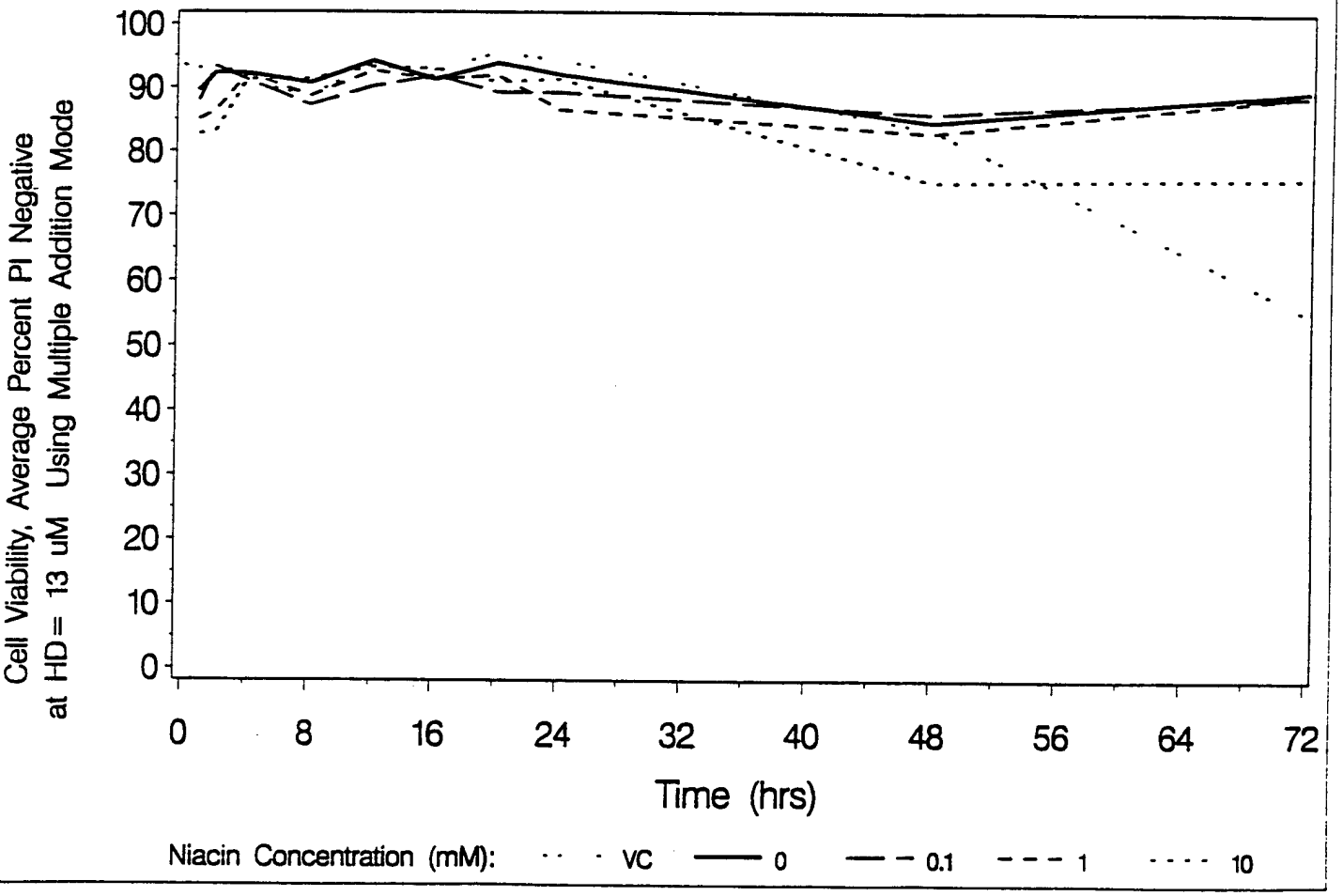


FIGURE J-7.

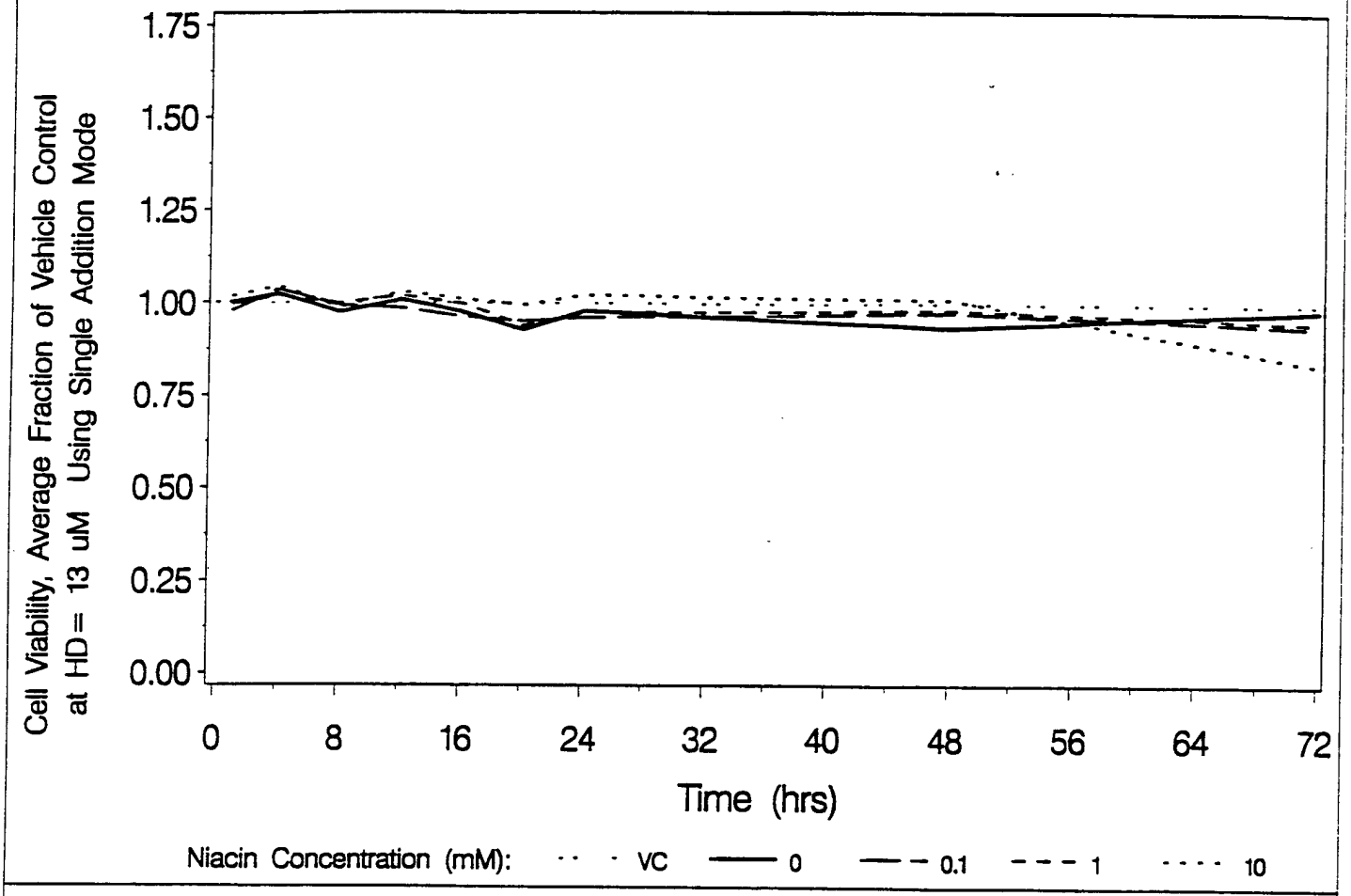


FIGURE J-8.

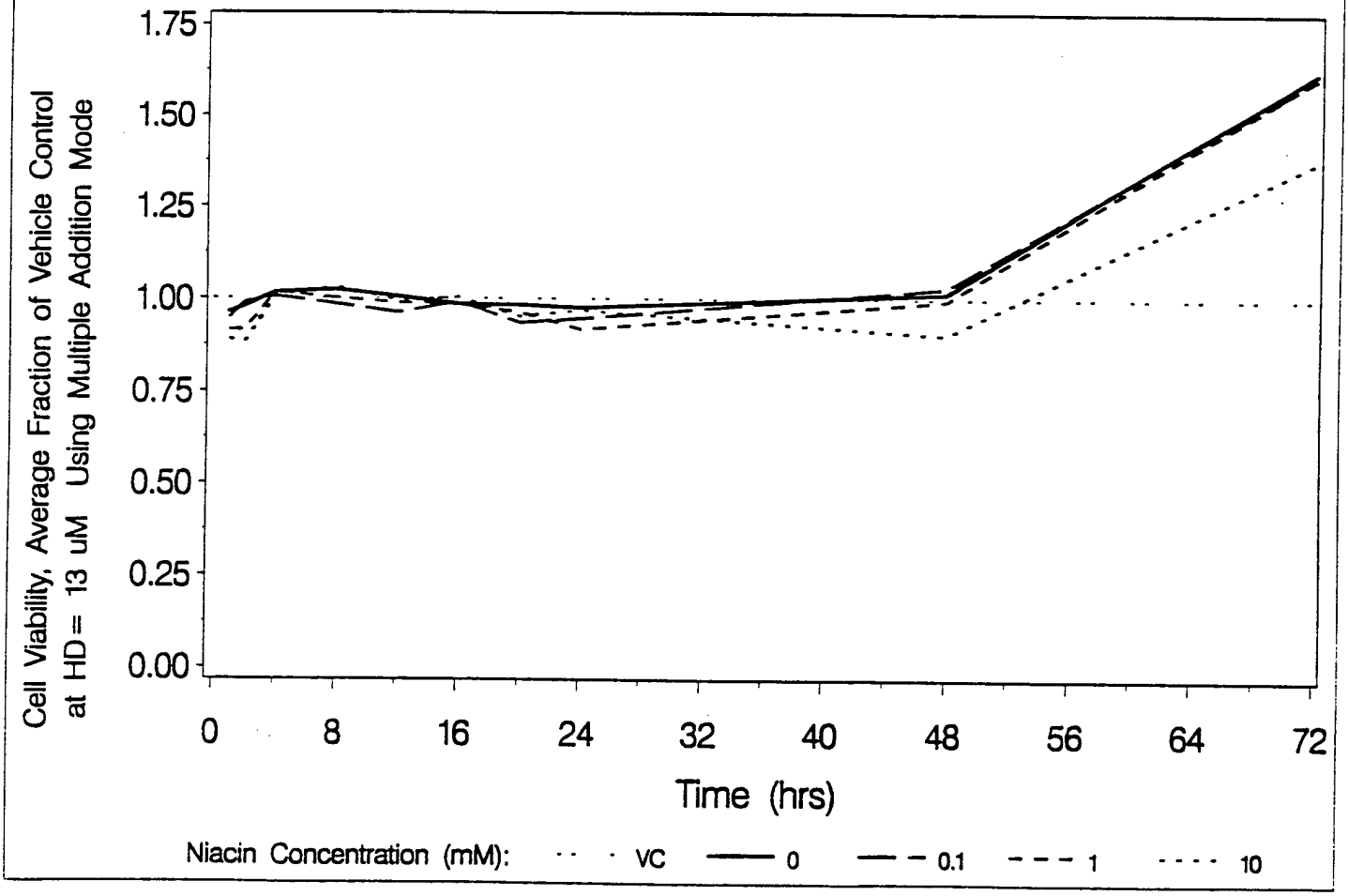


FIGURE J-9.

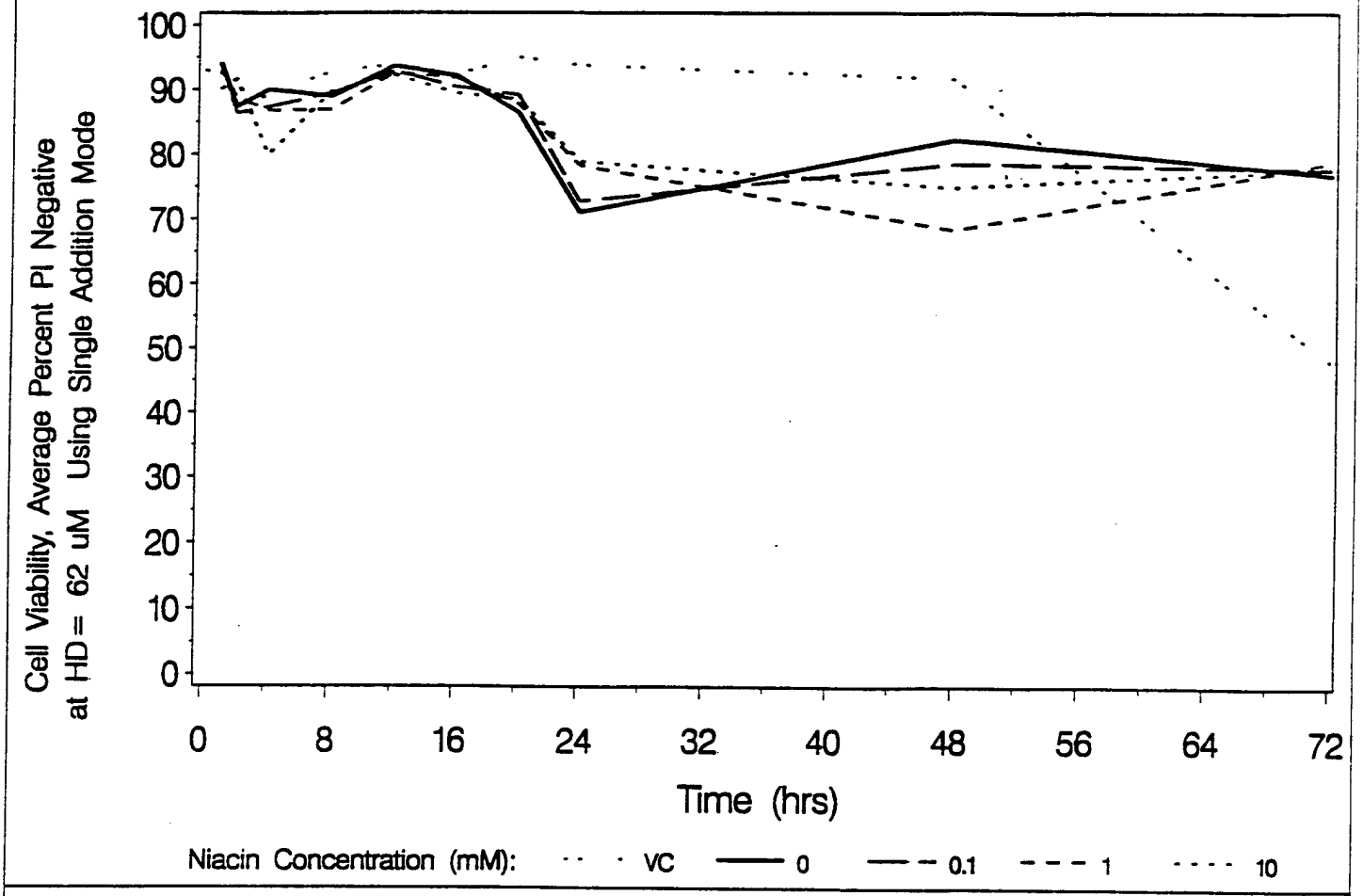


FIGURE J-10.

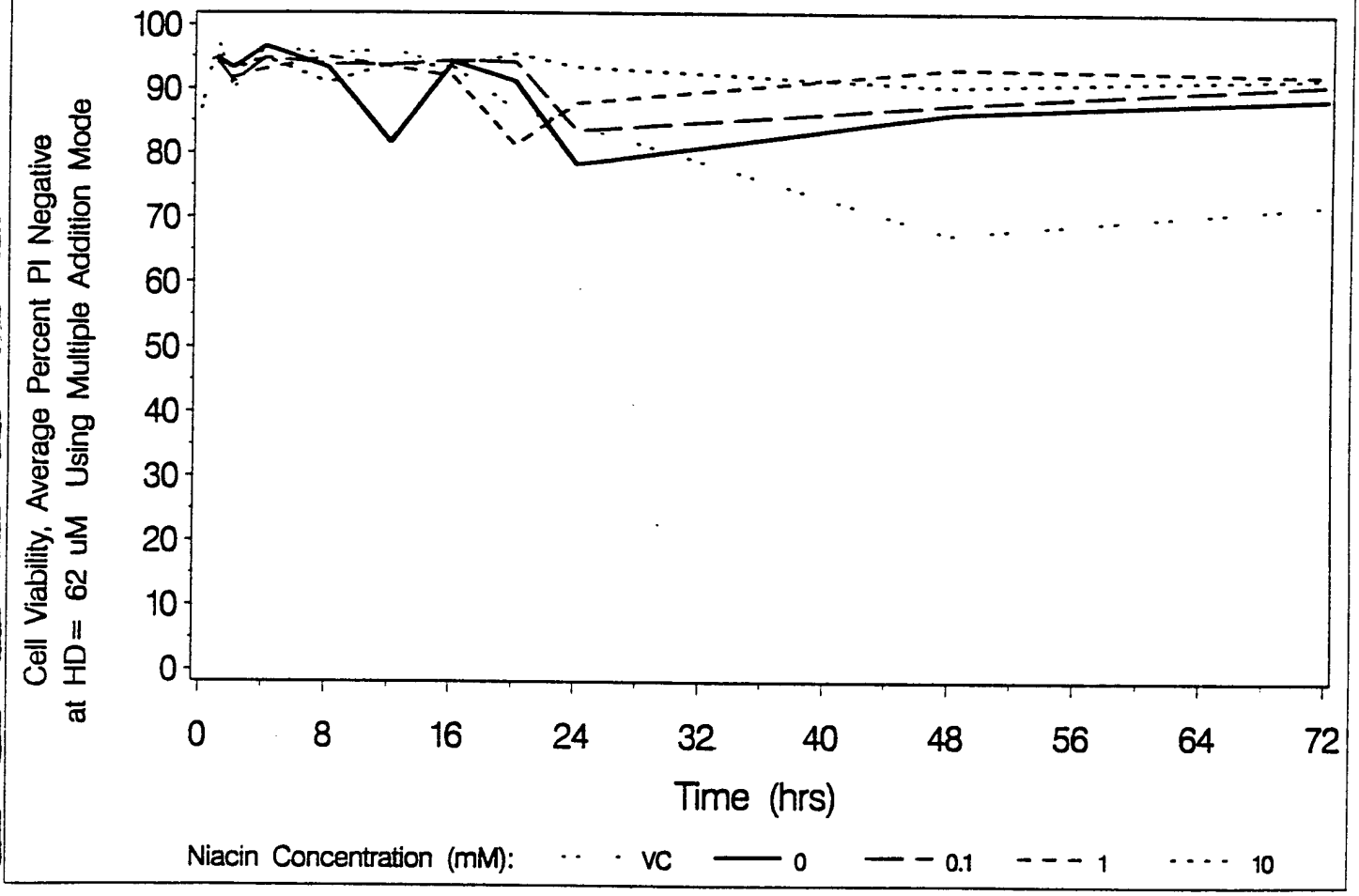


FIGURE J-11.

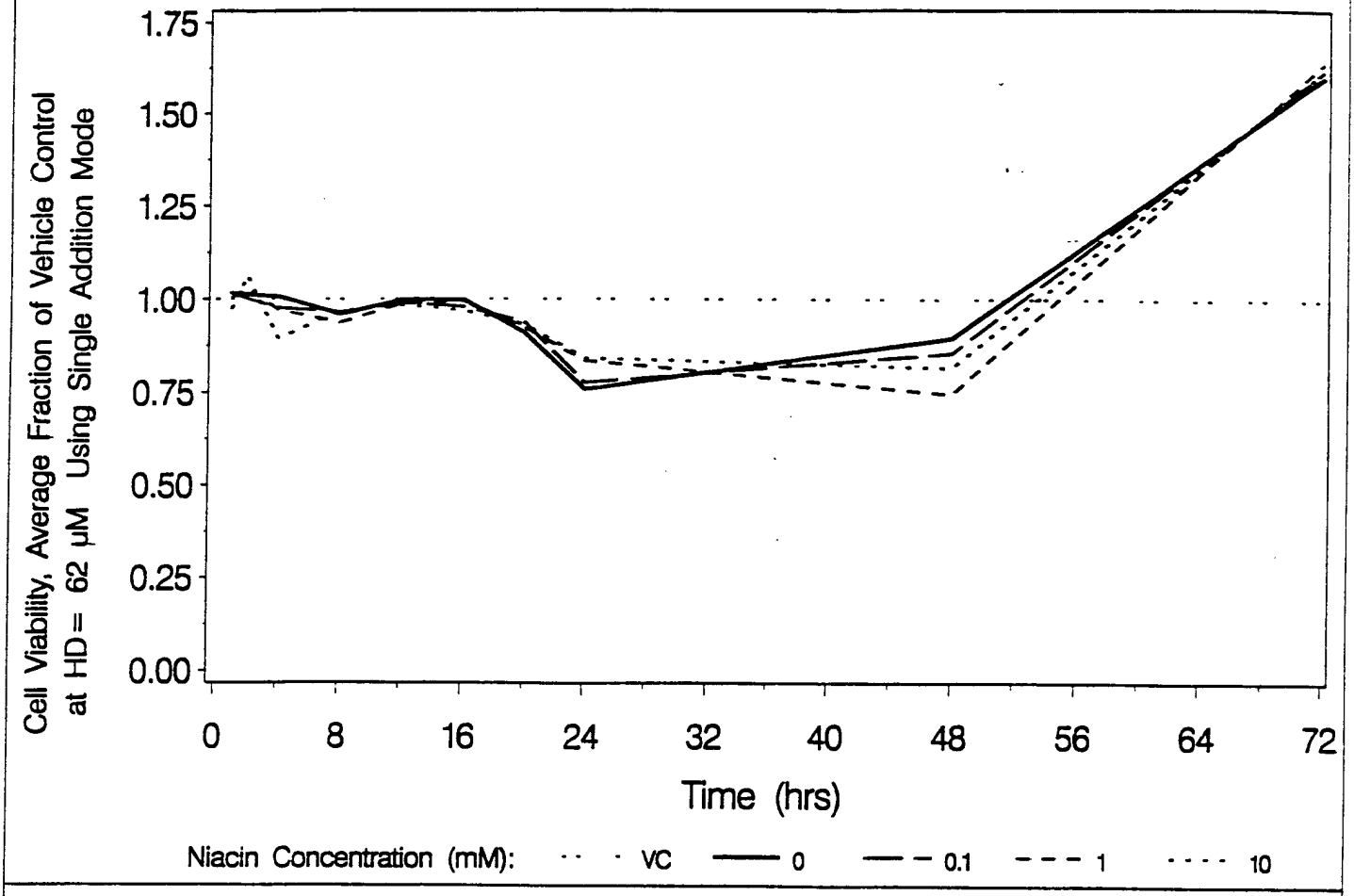


FIGURE J-12.

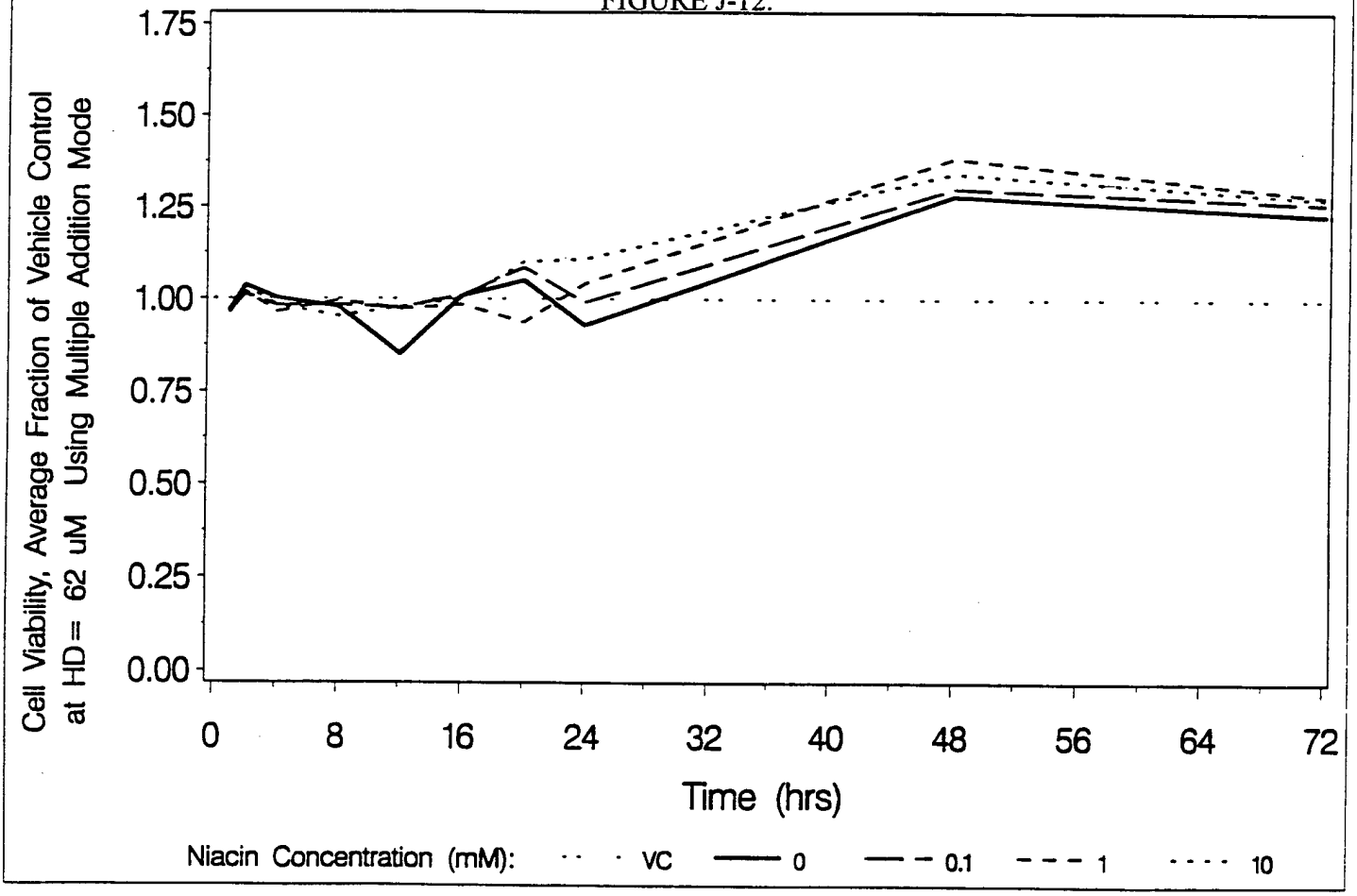


FIGURE J-13.

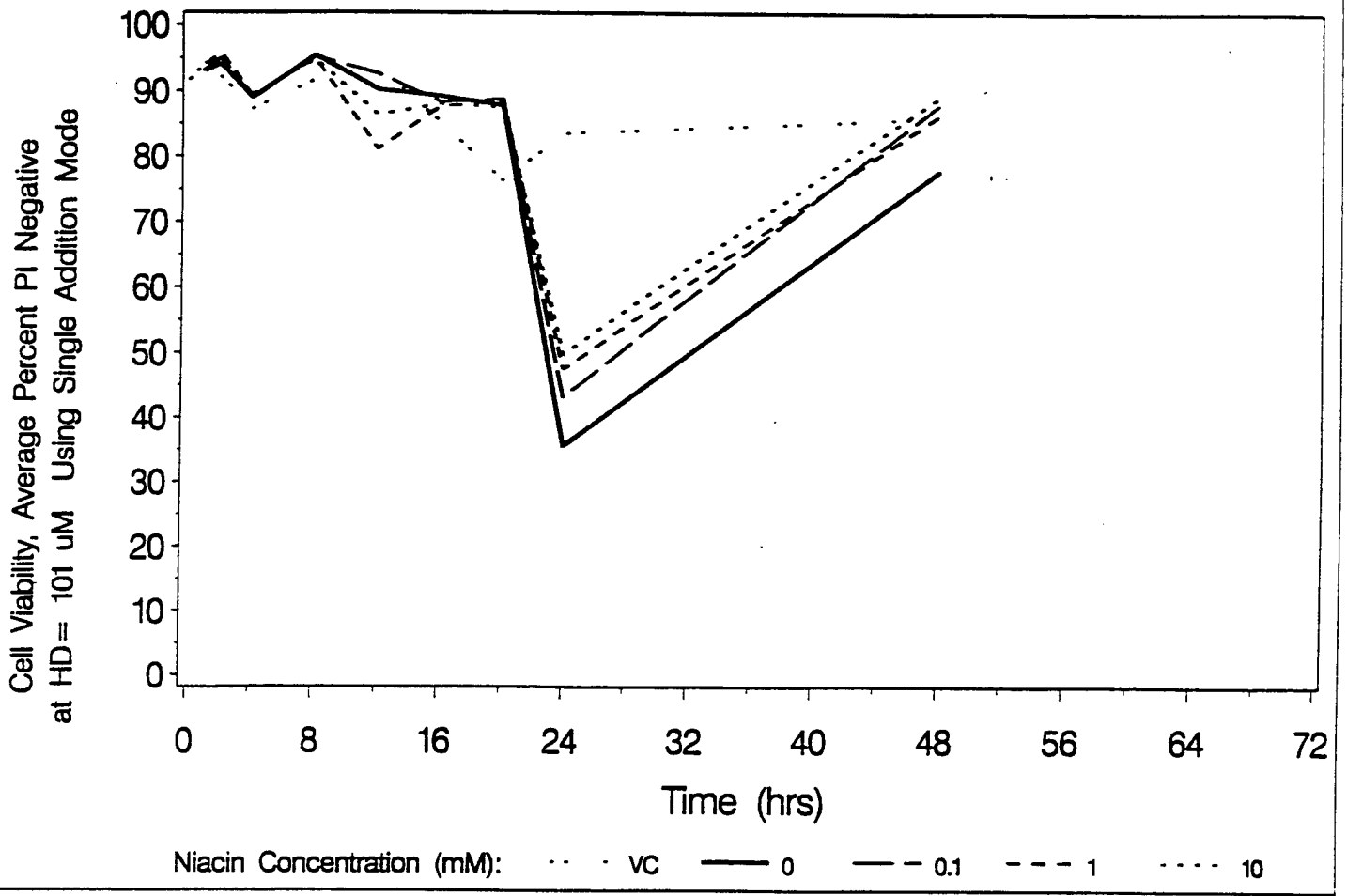


FIGURE J-14.

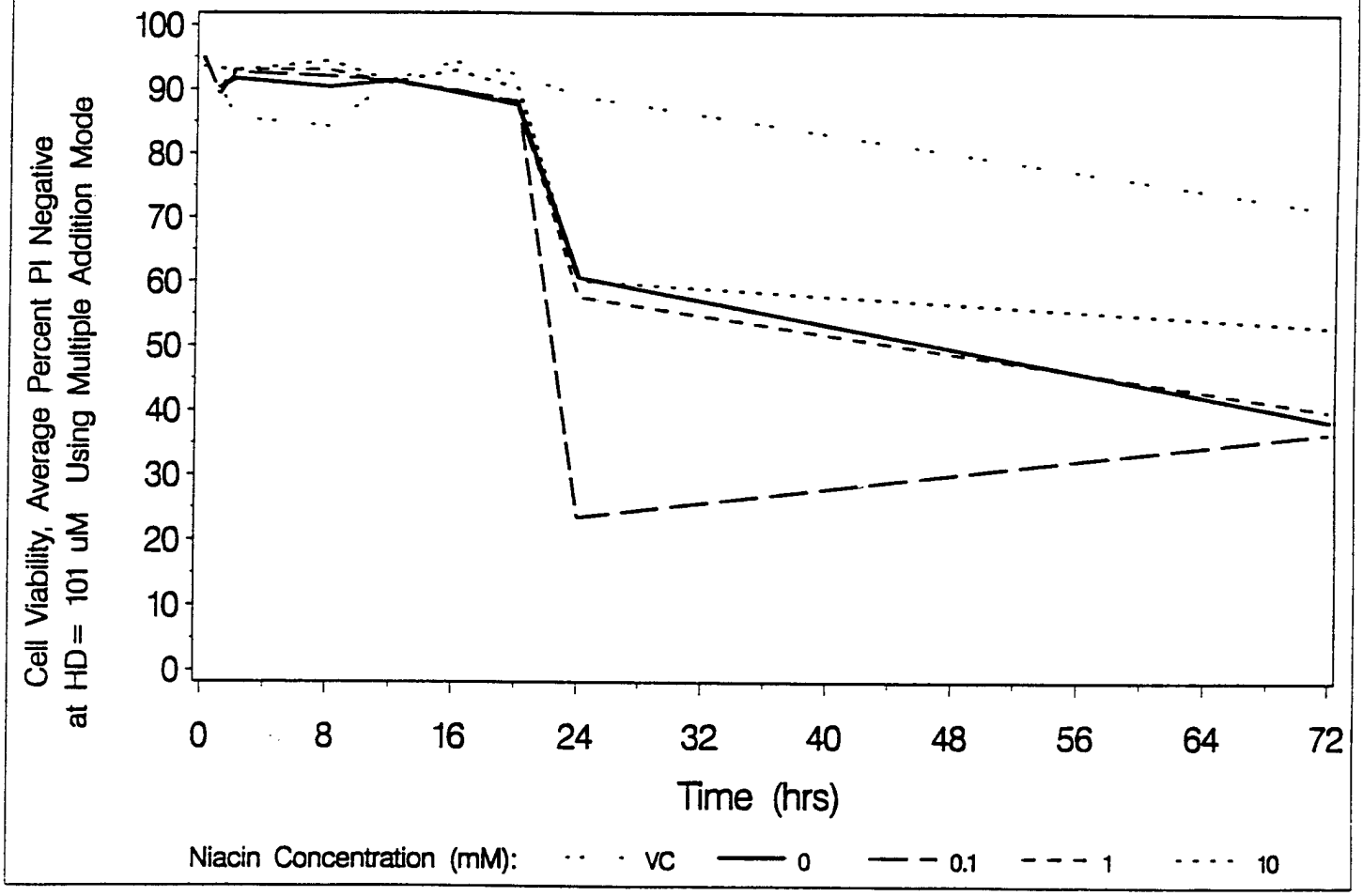


FIGURE J-15.

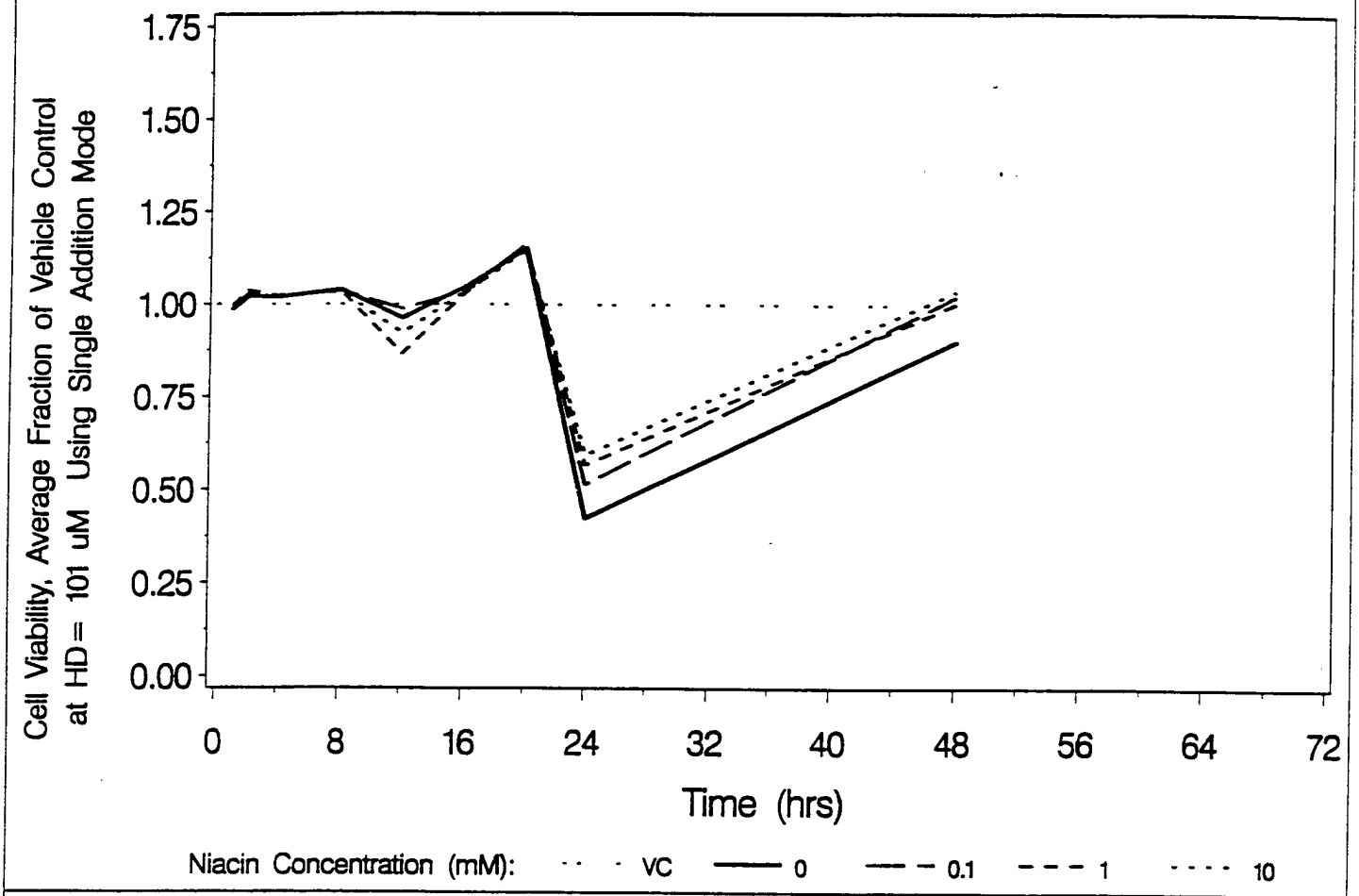


FIGURE J-16.

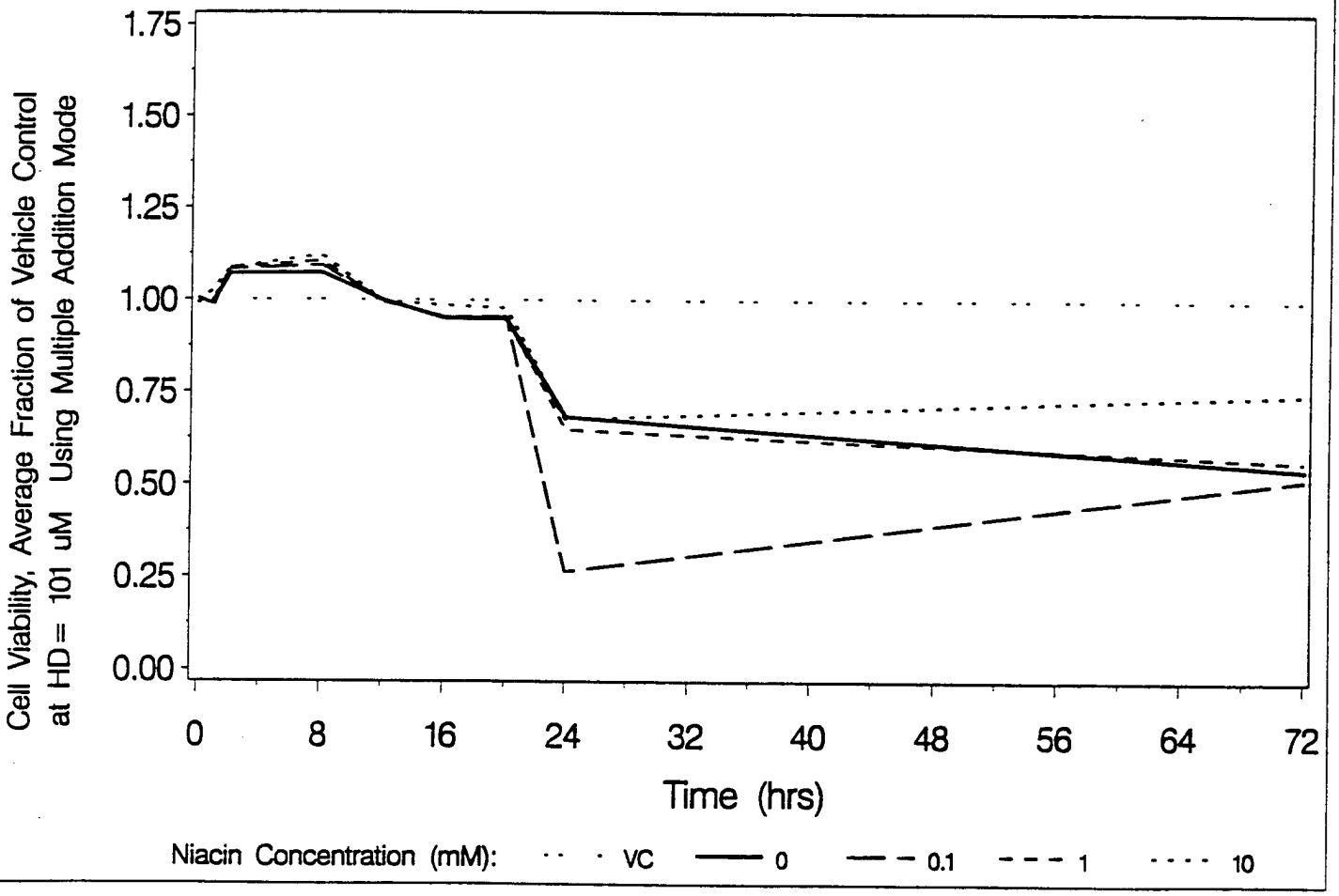


FIGURE J-17.

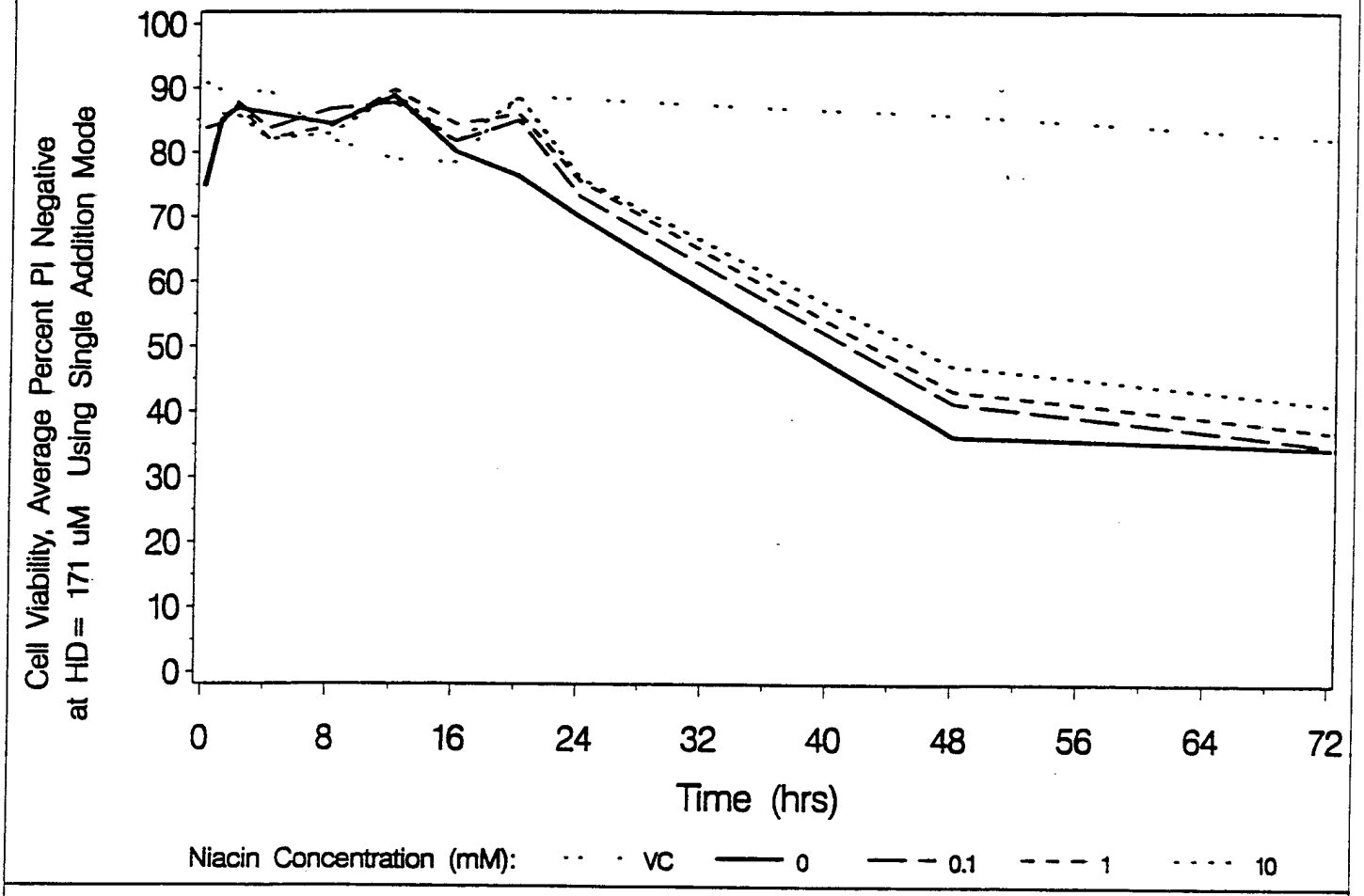


FIGURE J-18.

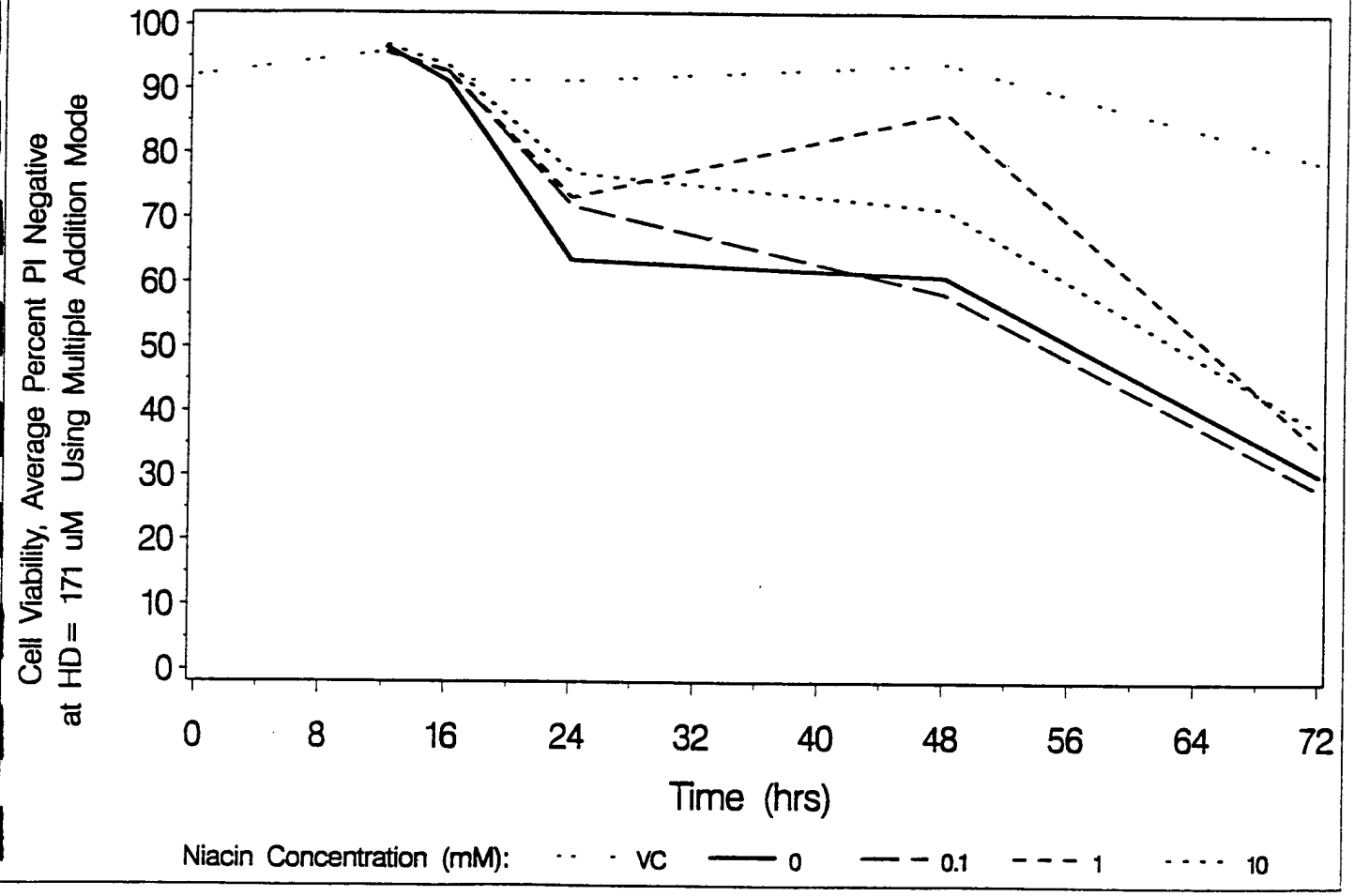


FIGURE J-19.

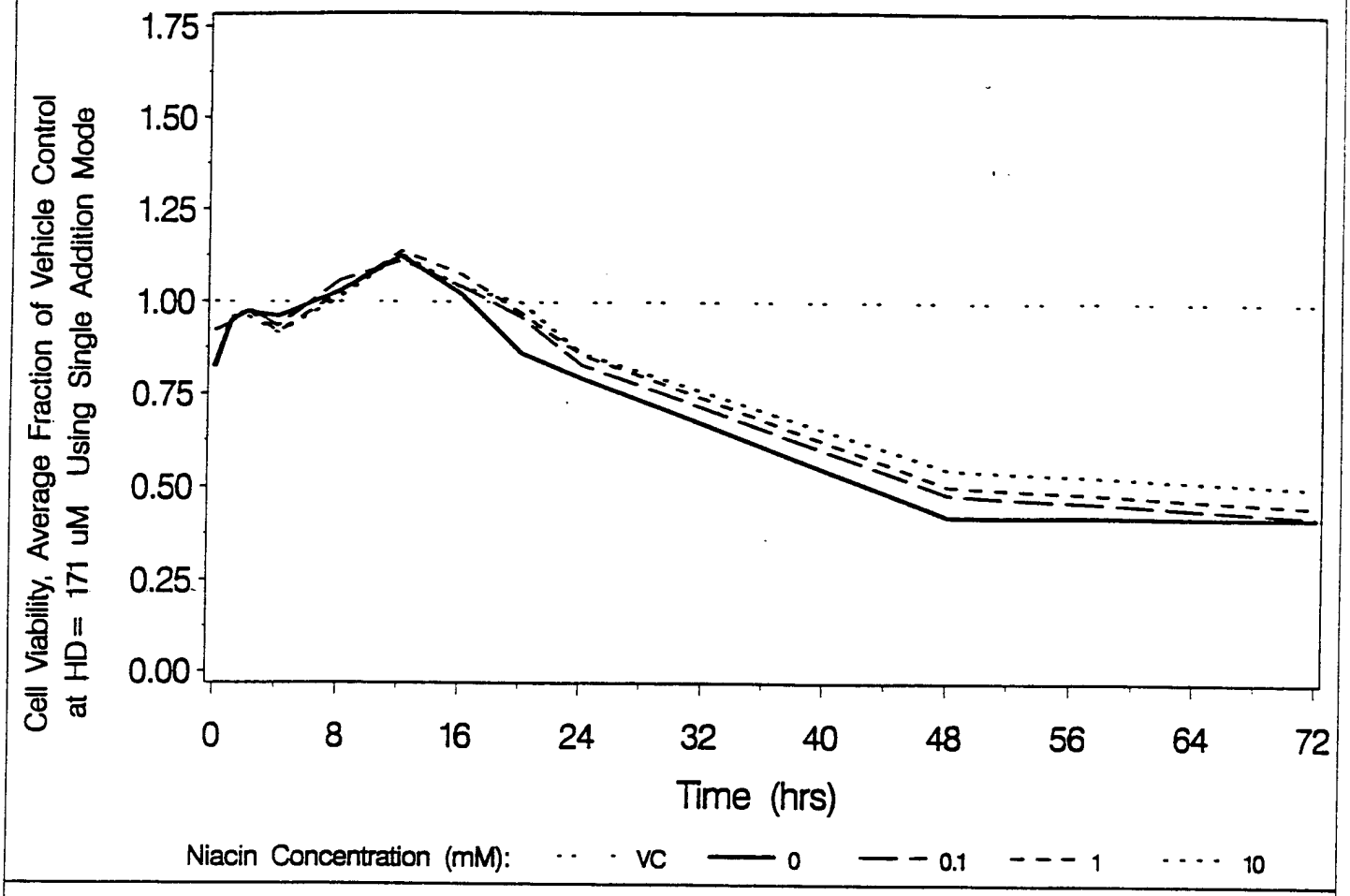
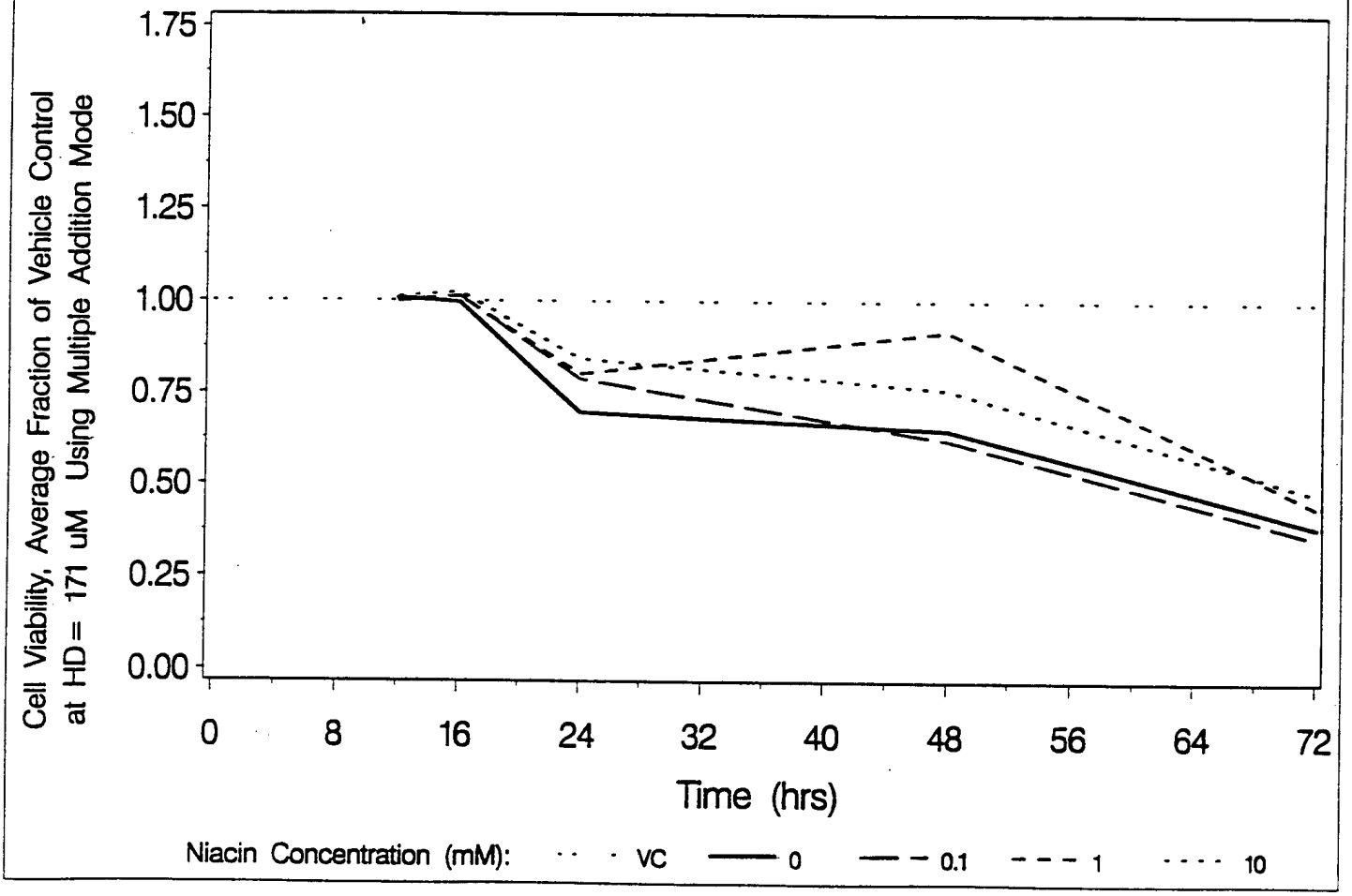


FIGURE J-20.



APPENDIX K

SUMMARY STATISTICS FOR CYTOTOXICITY DATA FROM NIACINAMIDE-PRETREATED, HD-EXPOSED CULTURES

TABLE K-1. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	90.0	4.6	3	91.3	4.7	3	80.1	9.1	3	86.6	3.3
	1	3	91.8	2.4	3	92.6	3.0	3	89.7	4.3	3	87.3	2.9
	4	3	89.1	2.4	3	84.0	4.2	3	84.5	0.3	3	85.5	3.2
	8	3	90.6	2.6	3	88.0	2.2	3	83.2	2.5	3	87.2	0.7
	12	3	92.0	2.7	3	93.9	0.5	3	94.7	1.0	3	94.3	2.6
	16	3	93.3	0.7	3	90.8	0.9	3	92.5	1.4	3	91.5	0.8
	20	3	91.0	1.0	3	90.6	2.3	3	87.7	1.2	3	87.1	2.9
	24	3	83.3	2.6	3	78.4	4.0	3	82.4	4.3	3	88.1	3.2
	48	3	85.3	1.3	3	84.2	2.0	3	86.8	3.2	3	89.0	0.3
	72	3	95.3	4.1	3	89.5	1.8	3	93.4	4.8	3	96.4	1.2
13	1	3	87.2	3.1	3	82.3	4.4	3	86.8	5.4	3	84.9	2.5
	4	3	87.5	0.5	3	88.3	2.8	3	87.5	3.2	3	86.1	4.8
	8	3	88.1	2.9	3	89.3	1.4	3	90.1	0.9	3	88.3	4.2
	12	3	92.8	1.9	3	90.8	2.2	3	93.9	1.7	3	94.8	2.8
	16	3	90.6	2.4	3	91.3	0.4	3	88.9	2.4	3	90.9	1.8
	20	3	86.0	0.5	3	89.7	1.1	3	88.6	0.9	3	89.6	1.1
	24	3	90.2	1.6	3	94.3	1.6	3	89.0	3.6	3	87.4	1.8
	48	3	84.8	1.9	3	88.5	2.2	3	80.3	1.4	3	85.3	1.5
	72	3	79.5	4.0	3	87.4	4.2	3	91.8	1.8	3	90.2	6.1

TABLE K-2. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.051	3	1.014	0.053	3	0.890	0.101	3	0.962	0.036
	1	3	1.000	0.027	3	1.009	0.033	3	0.977	0.047	3	0.951	0.031
	4	3	1.000	0.027	3	0.942	0.047	3	0.948	0.003	3	0.960	0.036
	8	3	1.000	0.029	3	0.971	0.024	3	0.918	0.028	3	0.962	0.008
	12	3	1.000	0.030	3	1.020	0.005	3	1.029	0.010	3	1.024	0.028
	16	3	1.000	0.007	3	0.973	0.010	3	0.991	0.015	3	0.981	0.009
	20	3	1.000	0.011	3	0.996	0.026	3	0.964	0.013	3	0.957	0.032
	24	3	1.000	0.031	3	0.942	0.048	3	0.990	0.051	3	1.058	0.039
	48	3	1.000	0.016	3	0.987	0.023	3	1.017	0.037	3	1.043	0.004
	72	3	1.000	0.043	3	0.939	0.019	3	0.979	0.050	3	1.011	0.013
13	1	3	0.950[a]	0.034	3	0.897	0.048	3	0.946	0.059	3	0.925	0.027
	4	3	0.982	0.006	3	0.991	0.032	3	0.982	0.036	3	0.966	0.054
	8	3	0.972	0.032	3	0.986	0.016	3	0.994	0.010	3	0.975	0.046
	12	3	1.009	0.020	3	0.986	0.024	3	1.020	0.019	3	1.030	0.030
	16	3	0.972	0.026	3	0.978	0.004	3	0.953	0.025	3	0.974	0.019
	20	3	0.945[a]	0.005	3	0.986[b]	0.012	3	0.974[b]	0.009	3	0.985[b]	0.012
	24	3	1.083[a]	0.019	3	1.133[b]	0.020	3	1.068	0.043	3	1.049	0.022
	48	3	0.994	0.022	3	1.038	0.026	3	0.941	0.016	3	1.000	0.017
	72	3	0.834[a]	0.042	3	0.917	0.044	3	0.963[b]	0.019	3	0.946[b]	0.064

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-3. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	93.5	1.3	-	-	-	-	-	-	-	-	-
	1	3	93.0	0.6	-	-	-	-	-	-	-	-	-
	2	3	94.3	0.9	-	-	-	-	-	-	-	-	-
	4	3	90.7	1.1	-	-	-	-	-	-	-	-	-
	8	3	88.7	0.8	-	-	-	-	-	-	-	-	-
	12	3	93.8	2.3	-	-	-	-	-	-	-	-	-
	16	3	92.7	1.3	-	-	-	-	-	-	-	-	-
	20	3	95.5	0.4	-	-	-	-	-	-	-	-	-
	24	3	94.3	1.0	-	-	-	-	-	-	-	-	-
	48	3	83.5	1.6	-	-	-	-	-	-	-	-	-
	72	3	55.3	2.9	-	-	-	-	-	-	-	-	-
13	1	3	89.5	0.2	3	91.6	1.8	3	92.7	1.6	3	88.8	4.0
	2	3	92.3	0.7	3	89.9	2.7	3	91.9	0.3	3	89.4	4.2
	4	3	92.2	0.8	3	93.1	1.8	3	94.1	0.6	3	93.5	0.8
	8	3	90.6	1.7	3	88.4	2.8	3	90.7	1.4	3	86.5	2.4
	12	3	94.2	0.3	3	92.2	3.1	3	92.9	0.6	3	91.8	1.7
	16	3	91.3	0.4	3	92.8	0.4	3	92.0	0.6	3	92.6	1.8
	20	3	93.9	1.1	3	94.2	2.4	3	94.9	1.7	3	94.3	0.5
	24	3	92.0	2.9	3	91.2	2.6	3	91.0	1.3	3	92.6	1.5
	48	3	84.7	2.4	3	82.1	6.2	3	82.1	2.1	3	82.9	2.3
	72	2	89.6	2.6	3	85.2	1.6	3	82.1	7.4	3	88.4	1.4

TABLE K-4. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.014	-	-	-	-	-	-	-	-	-
	1	3	1.000	0.006	-	-	-	-	-	-	-	-	-
	2	3	1.000	0.010	-	-	-	-	-	-	-	-	-
	4	3	1.000	0.012	-	-	-	-	-	-	-	-	-
	8	3	1.000	0.009	-	-	-	-	-	-	-	-	-
	12	3	1.000	0.025	-	-	-	-	-	-	-	-	-
	16	3	1.000	0.014	-	-	-	-	-	-	-	-	-
	20	3	1.000	0.004	-	-	-	-	-	-	-	-	-
	24	3	1.000	0.010	-	-	-	-	-	-	-	-	-
	48	3	1.000	0.019	-	-	-	-	-	-	-	-	-
	72	3	1.000	0.052	-	-	-	-	-	-	-	-	-
13	1	3	0.963[a]	0.002	3	0.985	0.019	3	0.997	0.018	3	0.955	0.043
	2	3	0.979	0.007	3	0.954	0.028	3	0.974	0.003	3	0.948	0.044
	4	3	1.017	0.009	3	1.027	0.020	3	1.037	0.007	3	1.030	0.009
	8	3	1.022	0.019	3	0.997	0.032	3	1.023	0.016	3	0.976	0.027
	12	3	1.004	0.003	3	0.983	0.033	3	0.990	0.007	3	0.978	0.018
	16	3	0.984	0.004	3	1.001	0.004	3	0.992	0.006	3	0.998	0.019
	20	3	0.983	0.011	3	0.986	0.025	3	0.993	0.017	3	0.988	0.005
	24	3	0.976	0.030	3	0.967	0.027	3	0.966	0.013	3	0.982	0.016
	48	3	1.015	0.029	3	0.984	0.075	3	0.983	0.026	3	0.993	0.027
	72	2	1.621[a]	0.046	3	1.541	0.030	3	1.485[b]	0.134	3	1.600	0.026

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-5. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	90.5	1.7	3	91.1	1.1	3	88.5	1.9	3	86.9	2.4
	1	3	92.2	1.0	3	93.4	1.8	3	89.1	1.3	3	90.7	0.3
	2	3	96.6	0.7	3	93.5	0.5	3	94.8	0.4	3	92.2	1.7
	4	3	94.0	2.0	3	92.6	0.7	3	88.1	2.5	3	90.4	1.2
	8	3	90.6	2.6	3	88.0	2.2	3	83.2	2.5	3	87.2	0.7
	12	3	91.2	1.3	3	94.1	1.7	3	90.1	3.2	3	89.6	2.5
	16	3	89.7	2.5	3	89.8	2.3	3	87.6	4.9	3	87.8	0.7
	20	3	87.4	2.9	3	89.2	2.3	3	88.9	2.6	3	88.8	1.4
	48	3	91.4	2.4	3	90.8	1.6	3	91.3	0.4	3	93.6	2.4
	72	3	96.7	2.7	3	91.5	2.0	3	91.5	1.9	3	95.4	2.1
62	1	3	89.1	2.0	3	90.2	1.9	3	85.5	1.2	3	87.9	2.5
	2	3	92.0	1.3	3	90.4	1.8	3	92.1	0.7	3	89.7	1.3
	4	3	90.7	1.4	3	88.6	1.5	3	87.5	3.0	3	88.7	1.3
	8	3	88.1	2.9	3	89.3	1.4	3	90.1	0.9	3	88.3	4.2
	12	3	87.2	2.2	3	84.8	10.6	3	86.0	2.9	3	87.8	4.2
	16	3	90.3	2.2	3	90.4	1.1	3	89.5	1.9	3	89.7	2.2
	20	3	86.2	4.1	3	86.9	0.6	3	85.9	0.2	3	86.8	2.1
	48	3	79.3	4.7	3	79.0	2.6	3	79.7	2.2	3	76.4	13.0
	72	3	76.8	3.1	3	82.5	4.3	3	76.2	3.6	3	76.0	6.7

TABLE K-6. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.019	3	1.006	0.013	3	0.978	0.021	3	0.960	0.026
	1	3	1.000	0.011	3	1.013	0.020	3	0.966	0.014	3	0.983	0.003
	2	3	1.000	0.007	3	0.968	0.006	3	0.982	0.004	3	0.955	0.018
	4	3	1.000	0.021	3	0.985	0.007	3	0.937	0.027	3	0.962	0.013
	8	3	1.000	0.029	3	0.971	0.024	3	0.918	0.028	3	0.962	0.008
	12	3	1.000	0.014	3	1.032	0.019	3	0.988	0.035	3	0.983	0.028
	16	3	1.000	0.028	3	1.001	0.026	3	0.977	0.054	3	0.979	0.008
	20	3	1.000	0.033	3	1.021	0.026	3	1.018	0.030	3	1.017	0.016
	48	3	1.000	0.027	3	0.994	0.017	3	0.999	0.005	3	1.024	0.026
	72	3	1.000	0.028	3	0.946	0.020	3	0.945	0.019	3	0.986	0.022
62	1	3	0.966	0.022	3	0.978	0.021	3	0.927[b]	0.013	3	0.953	0.027
	2	3	0.952[a]	0.013	3	0.936	0.018	3	0.954	0.007	3	0.929	0.013
	4	3	0.964	0.015	3	0.943	0.016	3	0.931[b]	0.032	3	0.944	0.014
	8	3	0.972	0.032	3	0.986	0.016	3	0.994	0.010	3	0.975	0.046
	12	3	0.957	0.025	3	0.930	0.117	3	0.943	0.031	3	0.963	0.046
	16	3	1.007	0.024	3	1.008	0.012	3	0.998	0.022	3	0.999	0.025
	20	3	0.986	0.046	3	0.995	0.007	3	0.984	0.003	3	0.994	0.024
	48	3	0.867[a]	0.052	3	0.865	0.028	3	0.872	0.024	3	0.836	0.142
	72	3	0.794[a]	0.032	3	0.853	0.044	3	0.788	0.037	3	0.786	0.069

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-7. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	92.1	2.6	3	93.8	0.5	3	92.4	1.3	3	94.9	2.5
	1	3	94.9	0.2	3	96.7	1.3	3	95.8	1.2	3	94.2	0.0
	2	3	91.2	2.5	3	96.0	0.8	3	95.6	0.9	3	92.8	1.0
	4	3	97.3	1.0	3	96.4	0.5	3	95.9	0.9	3	96.5	0.3
	8	3	94.9	2.1	3	95.6	0.1	3	95.2	1.9	3	95.2	1.8
	12	3	95.3	1.1	3	94.6	2.4	3	94.5	0.5	3	92.5	2.7
	16	3	95.1	3.2	3	94.7	2.2	3	91.3	4.6	3	93.7	2.3
	20	3	93.2	1.0	3	92.5	0.8	3	92.3	1.9	3	95.5	1.6
	24	3	91.3	0.6	3	92.6	4.5	3	87.0	5.6	3	88.3	4.6
	48	3	51.7	9.0	3	43.5	6.5	3	42.3	5.7	3	48.9	5.1
	72	3	73.2	13.1	3	63.4	1.7	3	46.9	8.0	3	51.2	6.9
62	1	3	94.9	0.8	3	92.4	1.6	3	91.9	1.4	3	93.6	1.1
	2	3	93.0	0.6	3	95.6	1.9	3	92.0	2.9	3	95.4	2.0
	4	3	96.3	0.4	3	96.4	0.6	3	96.9	1.4	3	96.4	0.4
	8	3	94.5	1.7	3	92.8	2.8	3	95.3	1.3	3	94.9	1.1
	12	3	95.1	0.6	3	94.8	0.7	3	93.9	0.8	3	93.4	2.8
	16	3	90.7	4.8	3	88.7	6.8	3	91.5	0.8	3	90.2	3.4
	20	3	95.3	2.0	3	94.5	1.8	3	94.1	0.9	3	92.9	0.3
	24	3	78.7	10.1	3	92.3	2.8	3	92.9	1.4	3	90.3	1.9
	48	3	81.9	2.5	3	82.5	1.0	3	82.3	1.4	3	86.0	2.1
	72	3	93.2	1.8	3	93.9	2.0	3	92.0	3.2	3	84.6	4.8

TABLE K-8. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.029	3	1.019	0.005	3	1.003	0.014	3	1.030	0.027
	1	3	1.000	0.002	3	1.019	0.014	3	1.009	0.013	3	0.993	0.000
	2	3	1.000	0.028	3	1.053	0.009	3	1.049	0.009	3	1.018	0.011
	4	3	1.000	0.010	3	0.991	0.005	3	0.986	0.009	3	0.992	0.003
	8	3	1.000	0.022	3	1.007	0.001	3	1.004	0.020	3	1.003	0.019
	12	3	1.000	0.011	3	0.993	0.025	3	0.992	0.005	3	0.971	0.029
	16	3	1.000	0.033	3	0.995	0.023	3	0.960	0.048	3	0.985	0.024
	20	3	1.000	0.011	3	0.992	0.009	3	0.991	0.021	3	1.024	0.018
	24	3	1.000	0.006	3	1.015	0.049	3	0.953	0.061	3	0.968	0.051
	48	3	1.000	0.175	3	0.841	0.125	3	0.819	0.111	3	0.946	0.099
	72	3	1.000	0.179	3	0.865	0.024	3	0.641	0.109	3	0.699	0.094
62	1	3	1.000	0.008	3	0.973	0.017	3	0.968[b]	0.015	3	0.987	0.012
	2	3	1.021	0.007	3	1.049	0.021	3	1.009	0.032	3	1.046	0.022
	4	3	0.990	0.004	3	0.991	0.006	3	0.996	0.014	3	0.991	0.004
	8	3	0.997	0.018	3	0.978	0.029	3	1.004	0.014	3	1.001	0.012
	12	3	0.999	0.006	3	0.995	0.007	3	0.985	0.008	3	0.981	0.029
	16	3	0.953	0.051	3	0.932	0.072	3	0.961	0.008	3	0.948	0.036
	20	3	1.022	0.022	3	1.014	0.019	3	1.010	0.010	3	0.997	0.003
	24	3	0.863[a]	0.111	3	1.011[b]	0.031	3	1.017[b]	0.015	3	0.990[b]	0.020
	48	3	1.585[a]	0.048	3	1.597	0.020	3	1.594	0.027	3	1.664	0.040
	72	3	1.273[a]	0.024	3	1.282	0.028	3	1.256	0.044	3	1.155[b]	0.065

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-9. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	88.7	2.0	-	-	-	-	-	-	-	-	-
	1	3	94.3	0.9	-	-	-	-	-	-	-	-	-
	2	3	88.9	2.9	-	-	-	-	-	-	-	-	-
	4	3	90.7	0.6	-	-	-	-	-	-	-	-	-
	8	3	93.5	2.3	-	-	-	-	-	-	-	-	-
	12	3	94.2	0.3	-	-	-	-	-	-	-	-	-
	16	3	93.9	0.3	-	-	-	-	-	-	-	-	-
	20	3	92.4	1.9	-	-	-	-	-	-	-	-	-
	24	3	91.9	2.2	-	-	-	-	-	-	-	-	-
	48	3	90.5	0.8	-	-	-	-	-	-	-	-	-
	72	3	79.5	5.1	-	-	-	-	-	-	-	-	-
101	1	3	92.7	3.0	3	92.3	2.1	3	90.7	2.5	3	90.8	0.9
	2	3	91.4	2.0	3	90.1	1.0	3	94.8	2.8	3	94.2	0.3
	4	3	92.0	1.3	3	89.9	3.7	3	89.2	1.4	3	90.5	1.2
	8	3	93.0	2.1	3	91.0	2.1	3	91.9	1.9	3	93.5	2.8
	12	3	95.0	0.8	3	94.2	0.3	3	93.0	1.9	3	94.3	0.6
	16	3	90.4	0.8	3	90.4	1.2	3	91.0	1.6	3	87.1	1.0
	20	3	66.7	1.2	3	63.7	1.7	3	64.9	3.8	3	63.7	3.4
	24	3	51.8	4.4	3	52.8	1.5	3	52.8	1.0	3	45.0	1.6
	48	3	26.6	2.0	3	28.1	1.9	3	25.6	1.9	3	26.0	2.3
	72	3	28.5	5.0	3	24.4	1.6	3	29.2	4.3	3	23.8	2.6

TABLE K-10. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.023	-	-	-	-	-	-	-	-	-
	1	3	1.000	0.009	-	-	-	-	-	-	-	-	-
	2	3	1.000	0.033	-	-	-	-	-	-	-	-	-
	4	3	1.000	0.007	-	-	-	-	-	-	-	-	-
	8	3	1.000	0.025	-	-	-	-	-	-	-	-	-
	12	3	1.000	0.003	-	-	-	-	-	-	-	-	-
	16	3	1.000	0.003	-	-	-	-	-	-	-	-	-
	20	3	1.000	0.021	-	-	-	-	-	-	-	-	-
	24	3	1.000	0.024	-	-	-	-	-	-	-	-	-
	48	3	1.000	0.009	-	-	-	-	-	-	-	-	-
	72	3	1.000	0.064	-	-	-	-	-	-	-	-	-
101	1	3	0.982	0.031	3	0.979	0.022	3	0.962	0.026	3	0.963	0.010
	2	3	1.029	0.022	3	1.014	0.011	3	1.067	0.032	3	1.060	0.004
	4	3	1.014	0.014	3	0.991	0.040	3	0.983	0.015	3	0.997	0.014
	8	3	0.995	0.023	3	0.973	0.022	3	0.983	0.020	3	1.000	0.030
	12	3	1.008	0.009	3	1.000	0.003	3	0.988	0.020	3	1.001	0.006
	16	3	0.963	0.009	3	0.963	0.013	3	0.969	0.017	3	0.928	0.010
	20	3	0.723[a]	0.013	3	0.690	0.019	3	0.703	0.042	3	0.689	0.037
	24	3	0.563[a]	0.048	3	0.574	0.017	3	0.574	0.011	3	0.489	0.018
	48	3	0.294[a]	0.022	3	0.311	0.021	3	0.283	0.021	3	0.287	0.025
	72	3	0.358[a]	0.063	3	0.306	0.021	3	0.367	0.053	3	0.299	0.033

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-11. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	93.2	1.3	3	92.6	0.9	3	93.7	0.8	3	93.0	0.4
	1	3	91.5	1.2	3	92.8	0.9	3	91.7	0.8	3	90.8	1.7
	2	3	86.5	3.6	3	84.3	3.6	3	82.9	3.9	3	81.1	2.3
	4	3	85.5	1.1	3	82.9	4.2	3	85.0	2.2	3	83.4	1.9
	12	3	89.8	0.4	3	89.1	4.7	3	91.2	2.2	3	92.1	1.3
	16	3	92.4	1.0	3	91.3	2.2	3	85.7	8.6	3	91.4	0.9
	20	3	90.9	1.8	3	93.2	0.4	3	92.2	0.6	3	93.8	1.2
	24	3	86.1	3.9	3	90.3	1.6	3	78.8	5.0	3	72.7	6.7
	48	3	87.8	2.1	3	86.5	3.3	3	85.4	0.8	3	80.6	3.3
	72	3	83.8	2.6	3	85.9	3.4	3	80.5	3.7	3	88.9	16.5
101	1	3	91.0	1.1	3	92.1	0.8	3	93.4	2.2	3	94.4	0.9
	2	3	85.1	4.0	3	87.6	4.7	3	89.0	2.6	3	93.2	0.6
	4	3	89.3	2.0	3	90.3	2.0	3	93.0	1.4	3	89.9	6.6
	12	3	89.2	3.8	3	89.7	3.0	3	90.1	2.1	3	91.3	2.0
	16	3	85.0	1.2	3	81.0	5.1	3	82.4	1.7	3	82.6	3.3
	20	3	81.6	4.2	3	81.4	3.3	3	83.1	1.7	3	81.0	3.5
	24	2	65.2	4.1	2	62.8	11.1	2	65.7	4.2	3	60.3	9.9
	48	3	38.2	1.4	3	40.3	3.0	3	36.3	1.5	3	38.9	11.4
	72	3	44.5	5.1	3	38.2	10.1	3	41.8	10.0	3	14.4	11.0

TABLE K-12. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.014	3	0.993	0.009	3	1.006	0.008	3	0.998	0.005
	1	3	1.000	0.013	3	1.014	0.010	3	1.002	0.009	3	0.991	0.018
	2	3	1.000	0.042	3	0.974	0.041	3	0.959	0.045	3	0.938	0.026
	4	3	1.000	0.013	3	0.970	0.049	3	0.994	0.026	3	0.976	0.022
	12	3	1.000	0.004	3	0.992	0.052	3	1.015	0.025	3	1.025	0.014
	16	3	1.000	0.011	3	0.989	0.024	3	0.928	0.093	3	0.989	0.010
	20	3	1.000	0.019	3	1.025	0.004	3	1.014	0.007	3	1.032	0.013
	24	3	1.000	0.045	3	1.049	0.019	3	0.915	0.058	3	0.844	0.077
	48	3	1.000	0.023	3	0.986	0.037	3	0.973	0.009	3	0.919	0.037
	72	3	1.000	0.031	3	1.024	0.040	3	0.960	0.045	3	1.061	0.197
101	1	3	0.994	0.012	3	1.007	0.008	3	1.021	0.024	3	1.031[b]	0.010
	2	3	0.984	0.047	3	1.013	0.055	3	1.029	0.030	3	1.077[b]	0.007
	4	3	1.044	0.024	3	1.057	0.024	3	1.088	0.017	3	1.052	0.077
	12	3	0.993	0.043	3	0.998	0.034	3	1.003	0.023	3	1.016	0.022
	16	3	0.921[a]	0.013	3	0.876	0.055	3	0.892	0.019	3	0.894	0.036
	20	3	0.897[a]	0.046	3	0.895	0.037	3	0.913	0.019	3	0.890	0.039
	24	2	0.758[a]	0.047	2	0.730	0.129	2	0.763	0.049	3	0.700	0.115
	48	3	0.435[a]	0.016	3	0.459	0.034	3	0.414	0.017	3	0.443	0.130
	72	3	0.531[a]	0.061	3	0.456	0.121	3	0.499	0.119	3	0.172[b]	0.132

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-13. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	2	3	77.9	4.5	3	78.9	2.9	3	63.6	8.6	3	66.3	14.8
	4	3	81.0	1.5	3	85.4	6.5	3	85.3	2.7	3	81.4	3.1
	8	3	88.6	2.9	3	86.9	0.9	3	89.4	2.5	3	87.4	1.0
	16	3	88.4	5.4	3	88.9	1.6	3	90.9	1.3	3	91.3	0.8
	20	3	91.8	0.2	3	90.2	1.4	3	90.2	1.0	3	92.1	1.2
	24	3	86.7	5.3	3	78.3	0.8	3	76.9	0.9	3	72.2	3.7
	48	3	86.5	2.5	3	81.6	0.7	3	83.9	7.3	3	72.6	8.3
171	2	3	89.3	1.6	3	85.9	3.0	3	90.1	1.9	3	90.9	1.1
	4	3	77.4	1.3	3	77.0	1.3	3	76.5	3.1	3	83.0	2.6
	8	3	88.4	1.2	3	87.0	1.2	3	85.7	1.8	3	85.8	1.8
	16	3	69.1	6.2	3	74.3	2.8	3	75.9	2.4	3	72.1	3.1
	20	3	67.6	2.5	3	68.3	2.4	3	71.5	4.5	3	69.4	3.7
	24	3	59.6	1.4	3	69.9	7.0	3	75.0	5.4	3	71.4	1.9
	48	3	20.4	4.4	3	25.5	5.4	3	35.7	4.8	3	24.9	4.4

TABLE K-14. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	2	3	1.000	0.058	3	1.012	0.037	3	0.817	0.110	3	0.851	0.191
	4	3	1.000	0.018	3	1.054	0.081	3	1.053	0.033	3	1.005	0.038
	8	3	1.000	0.032	3	0.981	0.010	3	1.009	0.029	3	0.987	0.011
	16	3	1.000	0.062	3	1.005	0.019	3	1.028	0.015	3	1.032	0.009
	20	3	1.000	0.002	3	0.983	0.015	3	0.983	0.011	3	1.004	0.013
	24	3	1.000	0.061	3	0.903	0.009	3	0.887	0.010	3	0.833	0.042
	48	3	1.000	0.029	3	0.942	0.008	3	0.969	0.085	3	0.838	0.096
171	2	3	1.146[a]	0.020	3	1.103	0.038	3	1.157	0.024	3	1.167	0.014
	4	3	0.955	0.016	3	0.950	0.016	3	0.945	0.038	3	1.024[b]	0.032
	8	3	0.997	0.013	3	0.981	0.013	3	0.968[b]	0.020	3	0.968[b]	0.021
	16	3	0.781[a]	0.070	3	0.840[b]	0.031	3	0.858[b]	0.027	3	0.815	0.036
	20	3	0.736[a]	0.028	3	0.744	0.026	3	0.779	0.049	3	0.756	0.040
	24	3	0.687[a]	0.016	3	0.806[b]	0.081	3	0.865[b]	0.062	3	0.823[b]	0.022
	48	3	0.236[a]	0.051	3	0.295	0.062	3	0.412[b]	0.055	3	0.288	0.051

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

TABLE K-15. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	2	3	82.0	3.6	3	82.7	0.7	3	84.6	1.1	3	90.0	1.0
	4	3	94.3	1.9	3	93.7	0.9	3	93.9	2.3	3	93.3	1.9
	8	3	97.1	0.7	3	96.5	0.5	3	95.9	1.4	3	96.3	0.7
	12	3	94.9	0.6	3	95.5	1.2	3	95.8	1.4	3	95.3	1.3
	16	3	91.4	3.2	3	92.8	2.4	3	91.3	4.2	3	87.8	3.7
	20	3	86.2	5.9	3	93.6	4.6	3	97.0	0.7	3	95.3	2.5
	24	3	94.0	2.7	3	94.9	1.4	3	97.8	0.7	3	87.2	8.4
	72	3	58.1	6.0	3	57.6	2.7	3	58.0	3.8	3	66.7	6.7
171	1	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	2	3	74.9	5.1	3	74.5	6.8	3	75.9	2.7	3	76.5	5.4
	4	3	93.9	1.8	3	94.5	0.9	3	95.2	1.3	3	94.4	1.3
	8	3	97.6	0.3	3	97.7	0.7	3	97.5	1.1	3	97.1	1.3
	12	3	95.9	0.4	3	95.1	1.3	3	95.3	1.5	3	94.3	1.4
	16	3	91.0	2.9	3	92.6	0.5	3	92.5	1.1	3	93.5	1.0
	20	3	96.8	0.6	3	94.3	2.5	3	96.1	0.3	3	95.8	0.8
	24	3	90.0	2.4	3	93.5	2.5	3	90.3	2.5	3	95.5	1.9
	72	3	40.9	16.9	3	39.0	7.0	3	50.8	8.4	3	45.8	10.5

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

TABLE K-16. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACINAMIDE (NM) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NM = 0 (mM)			NM = 0.01 (mM)			NM = 0.1 (mM)			NM = 1 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	1	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	2	3	1.000	0.045	3	1.009	0.008	3	1.031	0.013	3	1.098	0.013
	4	3	1.000	0.020	3	0.994	0.010	3	0.995	0.024	3	0.989	0.020
	8	3	1.000	0.007	3	0.995	0.005	3	0.988	0.015	3	0.992	0.008
	12	3	1.000	0.007	3	1.006	0.013	3	1.009	0.015	3	1.004	0.014
	16	3	1.000	0.035	3	1.016	0.026	3	0.999	0.045	3	0.961	0.041
	20	3	1.000	0.068	3	1.085	0.054	3	1.125	0.008	3	1.106	0.030
	24	3	1.000	0.029	3	1.010	0.015	3	1.041	0.008	3	0.928	0.090
	72	3	1.000	0.103	3	0.992	0.046	3	0.999	0.065	3	1.148	0.114
171	1	0*	-	-	0*	-	-	0*	-	-	0*	-	-
	2	3	0.913	0.062	3	0.908	0.082	3	0.926	0.033	3	0.933	0.066
	4	3	0.996	0.019	3	1.001	0.009	3	1.009	0.014	3	1.001	0.014
	8	3	1.005	0.003	3	1.006	0.007	3	1.005	0.012	3	1.000	0.013
	12	3	1.010	0.005	3	1.002	0.013	3	1.004	0.015	3	0.993	0.014
	16	3	0.996	0.032	3	1.014	0.005	3	1.012	0.012	3	1.023	0.011
	20	3	1.122	0.006	3	1.094	0.029	3	1.115	0.003	3	1.112	0.009
	24	3	0.958	0.025	3	0.995	0.026	3	0.961	0.026	3	1.016	0.020
	72	3	0.703[a]	0.292	3	0.671	0.120	3	0.874	0.144	3	0.788	0.181

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NM = 0) value.

[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NM = 0) value.

APPENDIX L

SUMMARY STATISTICS FOR CYTOTOXICITY DATA FROM NIACIN-PRETREATED, HD-EXPOSED CULTURES

TABLE L-1. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	87.9	4.9	3	74.5	2.0	3	80.6	5.5	3	83.1	6.8
	1	3	91.1	2.3	3	90.2	1.8	3	90.1	2.0	2	90.7	0.3
	4	3	86.0	1.9	3	89.2	3.0	3	85.7	2.2	3	86.4	4.0
	8	3	85.2	1.2	3	83.1	5.3	3	83.1	1.1	3	84.6	4.6
	12	3	92.0	2.7	3	93.9	0.5	3	94.7	1.0	3	94.3	2.6
	16	3	91.8	0.3	3	90.2	1.8	3	90.2	1.1	3	91.9	2.4
	20	3	92.2	1.0	3	90.7	2.1	3	88.7	1.8	3	90.8	2.0
	24	3	86.8	2.3	3	84.7	1.5	3	82.0	1.9	3	87.9	2.3
	48	3	89.4	2.3	3	91.3	1.6	3	92.3	2.2	3	92.3	0.7
	72	3	91.6	2.9	3	93.5	0.9	3	92.1	2.0	3	92.9	1.8
13	1	3	91.2	1.5	3	89.1	3.6	3	90.7	2.3	3	92.6	0.6
	4	3	88.1	1.8	3	89.2	2.4	3	87.7	1.6	3	90.0	0.7
	8	3	83.0	5.1	3	84.7	0.2	3	85.1	1.8	3	84.1	0.7
	12	3	92.8	1.9	3	90.8	2.2	3	93.9	1.7	3	94.8	2.8
	16	3	89.6	0.9	3	88.4	2.1	3	91.6	1.5	3	92.8	2.2
	20	3	85.6	1.9	3	87.8	0.3	3	86.8	1.8	3	91.7	1.0
	24	3	85.0	5.2	3	83.4	6.6	3	84.5	4.4	3	88.7	4.3
	48	3	83.6	2.5	3	87.2	1.3	3	87.9	1.1	3	90.3	0.8
	72	3	90.2	1.2	3	86.2	2.8	3	87.2	0.6	3	76.8	14.0

TABLE L-2. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	2	1.000	0.056	3	0.848	0.022	3	0.917	0.062	3	0.946	0.078
	1	3	1.000	0.026	3	0.991	0.019	3	0.990	0.021	2	0.996	0.003
	4	3	1.000	0.022	3	1.037	0.035	3	0.996	0.026	3	1.004	0.047
	8	3	1.000	0.014	3	0.975	0.063	3	0.975	0.013	3	0.993	0.054
	12	3	1.000	0.030	3	1.020	0.005	3	1.029	0.010	3	1.024	0.028
	16	3	1.000	0.003	3	0.983	0.019	3	0.983	0.012	3	1.001	0.026
	20	3	1.000	0.011	3	0.983	0.023	3	0.962	0.019	3	0.985	0.022
	24	3	1.000	0.027	3	0.976	0.017	3	0.945	0.022	3	1.013	0.027
	48	3	1.000	0.026	3	1.022	0.018	3	1.033	0.025	3	1.033	0.008
	72	3	1.000	0.031	3	1.020	0.009	3	1.005	0.022	3	1.014	0.020
13	1	3	1.001	0.017	3	0.978	0.040	3	0.996	0.025	3	1.017	0.007
	4	3	1.024	0.020	3	1.037	0.028	3	1.020	0.019	3	1.046	0.008
	8	3	0.975	0.060	3	0.994	0.002	3	0.999	0.021	3	0.987	0.008
	12	3	1.009	0.020	3	0.986	0.024	3	1.020	0.019	3	1.030	0.030
	16	3	0.976	0.010	3	0.963	0.023	3	0.998	0.016	3	1.011[b]	0.024
	20	3	0.929[a]	0.020	3	0.952	0.003	3	0.941	0.019	3	0.995[b]	0.011
	24	3	0.980	0.060	3	0.961	0.076	3	0.974	0.051	3	1.022	0.049
	48	3	0.935[a]	0.028	3	0.975	0.014	3	0.983	0.012	3	1.011	0.009
	72	3	0.985	0.013	3	0.941	0.030	3	0.952	0.007	3	0.838	0.153

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-3. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	93.5	1.3	-	-	-	-	-	-	-	-	-
	1	3	93.0	0.6	-	-	-	-	-	-	-	-	-
	2	3	94.3	0.9	-	-	-	-	-	-	-	-	-
	4	3	90.7	1.1	-	-	-	-	-	-	-	-	-
	8	3	88.7	0.8	-	-	-	-	-	-	-	-	-
	12	3	93.8	2.3	-	-	-	-	-	-	-	-	-
	16	3	92.7	1.3	-	-	-	-	-	-	-	-	-
	20	3	95.5	0.4	-	-	-	-	-	-	-	-	-
	24	3	94.3	1.0	-	-	-	-	-	-	-	-	-
	48	3	83.5	1.6	-	-	-	-	-	-	-	-	-
	72	3	55.3	2.9	-	-	-	-	-	-	-	-	-
13	1	3	89.5	0.2	3	88.0	2.9	3	85.0	1.5	3	82.7	5.2
	2	3	92.3	0.7	3	93.3	1.3	3	86.3	0.4	3	83.0	1.3
	4	3	92.2	0.8	3	91.1	1.9	3	92.2	2.4	3	91.3	1.8
	8	3	90.6	1.7	3	87.2	0.9	3	88.6	0.8	3	91.3	2.5
	12	3	94.2	0.3	3	90.2	3.1	3	92.6	1.4	3	93.2	1.0
	16	3	91.3	0.4	3	91.8	1.1	3	91.3	0.5	3	93.0	0.3
	20	3	93.9	1.1	3	89.3	3.1	3	92.0	1.2	3	90.9	5.2
	24	3	92.0	2.9	3	89.2	1.8	3	86.5	6.2	3	91.3	0.2
	48	3	84.7	2.4	3	86.0	1.6	3	83.0	6.5	3	75.3	23.2
	72	2	89.6	2.6	3	88.9	1.0	3	89.0	3.4	3	76.0	30.4

TABLE L-4. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 13 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.014	-	-	-	-	-	-	-	-	-
	1	3	1.000	0.006	-	-	-	-	-	-	-	-	-
	2	3	1.000	0.010	-	-	-	-	-	-	-	-	-
	4	3	1.000	0.012	-	-	-	-	-	-	-	-	-
	8	3	1.000	0.009	-	-	-	-	-	-	-	-	-
	12	3	1.000	0.025	-	-	-	-	-	-	-	-	-
	16	3	1.000	0.014	-	-	-	-	-	-	-	-	-
	20	3	1.000	0.004	-	-	-	-	-	-	-	-	-
	24	3	1.000	0.010	-	-	-	-	-	-	-	-	-
	48	3	1.000	0.019	-	-	-	-	-	-	-	-	-
	72	3	1.000	0.052	-	-	-	-	-	-	-	-	-
13	1	3	0.963[a]	0.002	3	0.947	0.031	3	0.915	0.017	3	0.889[b]	0.056
	2	3	0.979	0.007	3	0.990	0.013	3	0.915[b]	0.005	3	0.881[b]	0.013
	4	3	1.017	0.009	3	1.005	0.021	3	1.016	0.027	3	1.007	0.019
	8	3	1.022	0.019	3	0.984	0.011	3	0.999	0.009	3	1.030	0.028
	12	3	1.004	0.003	3	0.961[b]	0.033	3	0.987	0.015	3	0.994	0.010
	16	3	0.984	0.004	3	0.990	0.012	3	0.984	0.006	3	1.003	0.004
	20	3	0.983	0.011	3	0.935[b]	0.033	3	0.963	0.012	3	0.952	0.055
	24	3	0.976	0.030	3	0.946	0.020	3	0.918	0.066	3	0.969	0.003
	48	3	1.015	0.029	3	1.030	0.019	3	0.994	0.077	3	0.903	0.278
	72	2	1.621[a]	0.046	3	1.608	0.018	3	1.610	0.062	3	1.375	0.549

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-5. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	93.0	1.0	-	-	-	-	-	-	-	-	-
	1	3	92.5	0.9	-	-	-	-	-	-	-	-	-
	2	3	86.2	3.8	-	-	-	-	-	-	-	-	-
	4	3	89.3	2.3	-	-	-	-	-	-	-	-	-
	8	3	92.8	1.7	-	-	-	-	-	-	-	-	-
	12	3	93.9	1.3	-	-	-	-	-	-	-	-	-
	16	3	92.3	0.2	-	-	-	-	-	-	-	-	-
	20	3	94.9	1.2	-	-	-	-	-	-	-	-	-
	24	3	93.7	0.1	-	-	-	-	-	-	-	-	-
	48	3	91.7	0.8	-	-	-	-	-	-	-	-	-
	72	3	48.3	1.6	-	-	-	-	-	-	-	-	-
62	1	3	94.0	1.3	2	94.1	1.3	3	92.8	1.0	3	90.1	1.0
	2	3	87.3	2.0	2	86.3	2.8	3	88.7	1.0	3	91.6	2.6
	4	3	89.9	1.3	3	87.3	4.4	3	86.7	3.1	3	79.8	1.4
	8	3	88.9	0.9	3	89.4	1.5	3	86.9	3.8	3	89.8	4.4
	12	3	93.7	1.4	3	92.9	0.2	3	92.6	1.4	3	92.3	2.0
	16	3	92.1	2.2	3	90.4	1.6	3	91.8	0.9	3	89.4	2.0
	20	3	86.3	1.0	3	89.1	0.7	3	87.7	0.5	3	88.4	1.1
	24	3	71.0	18.6	3	72.7	1.8	3	78.1	0.7	3	78.7	0.6
	48	3	82.2	2.7	3	78.5	1.1	3	68.4	7.2	3	74.9	3.5
	72	3	77.1	5.5	3	77.9	3.0	3	79.0	2.3	3	78.0	5.0

TABLE L-6. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.011	-	-	-	-	-	-	-	-	-
	1	3	1.000	0.010	-	-	-	-	-	-	-	-	-
	2	3	1.000	0.044	-	-	-	-	-	-	-	-	-
	4	3	1.000	0.026	-	-	-	-	-	-	-	-	-
	8	3	1.000	0.018	-	-	-	-	-	-	-	-	-
	12	3	1.000	0.014	-	-	-	-	-	-	-	-	-
	16	3	1.000	0.003	-	-	-	-	-	-	-	-	-
	20	3	1.000	0.013	-	-	-	-	-	-	-	-	-
	24	3	1.000	0.001	-	-	-	-	-	-	-	-	-
	48	3	1.000	0.008	-	-	-	-	-	-	-	-	-
	72	3	1.000	0.033	-	-	-	-	-	-	-	-	-
62	1	3	1.016	0.014	2	1.018	0.014	3	1.004	0.011	3	0.974[b]	0.011
	2	3	1.012	0.023	2	1.001	0.033	3	1.029	0.012	3	1.063[b]	0.030
	4	3	1.007	0.015	3	0.977	0.049	3	0.971	0.035	3	0.894[b]	0.016
	8	3	0.959	0.010	3	0.964	0.016	3	0.937	0.041	3	0.968	0.048
	12	3	0.998	0.015	3	0.989	0.003	3	0.987	0.014	3	0.983	0.021
	16	3	0.997	0.024	3	0.979	0.018	3	0.994	0.010	3	0.968	0.021
	20	3	0.909[a]	0.010	3	0.938	0.007	3	0.924	0.005	3	0.931	0.012
	24	3	0.757[a]	0.199	3	0.775	0.020	3	0.834	0.007	3	0.840	0.007
	48	3	0.897[a]	0.029	3	0.857	0.012	3	0.746[b]	0.078	3	0.817	0.038
	72	3	1.597[a]	0.113	3	1.614	0.062	3	1.636	0.048	3	1.616	0.103

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-7. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	86.8	2.2	3	82.6	4.4	3	90.1	2.4	3	90.2	3.6
	1	3	97.8	1.1	3	96.8	0.5	3	96.8	0.1	3	95.0	1.1
	2	3	89.9	2.5	3	91.5	0.9	3	88.6	2.4	3	91.7	1.5
	4	3	96.5	0.8	3	95.4	0.5	3	95.6	0.3	3	95.9	1.0
	8	3	95.4	0.2	3	95.3	1.6	3	95.3	0.9	3	94.8	1.2
	12	3	96.0	1.2	3	94.5	0.9	3	94.7	1.1	3	94.9	1.3
	16	3	93.4	0.8	3	93.7	0.4	3	93.9	1.7	3	94.5	2.1
	20	3	86.7	9.1	3	92.7	4.9	3	96.1	1.3	1	94.3	-
	24	3	84.3	2.0	3	87.6	2.3	3	85.9	2.5	3	88.2	4.1
	48	3	67.3	9.5	1	75.7	-	3	65.5	2.7	3	91.7	2.1
	72	3	72.1	10.8	3	61.5	12.7	3	62.9	6.7	3	73.4	3.2
62	1	3	94.7	1.6	3	94.4	1.3	3	94.0	0.9	3	95.1	0.4
	2	3	93.2	0.7	3	91.0	1.8	3	91.6	1.4	3	92.9	1.2
	4	3	96.6	0.3	3	94.8	0.7	3	93.0	0.7	3	94.8	2.0
	8	3	93.2	0.2	3	93.8	0.6	3	94.9	1.5	3	90.8	6.8
	12	3	81.6	20.8	3	93.7	1.1	3	93.4	1.4	3	93.9	1.6
	16	3	94.1	0.9	3	94.4	0.4	3	91.8	3.4	3	93.4	2.0
	20	3	91.1	3.3	3	94.1	4.4	3	81.2	16.3	3	95.3	1.1
	24	3	78.2	1.1	3	83.4	1.6	3	87.8	2.8	3	93.4	2.4
	48	3	86.2	3.4	3	87.6	4.6	1	93.2	-	2	90.4	1.0
	72	3	88.7	0.7	3	90.9	1.6	3	92.4	1.9	3	91.9	1.9

TABLE L-8. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 62 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.025	3	0.951	0.051	3	1.037	0.027	3	1.039	0.041
	1	3	1.000	0.011	3	0.991	0.006	3	0.991	0.001	3	0.972	0.011
	2	3	1.000	0.028	3	1.018	0.010	3	0.987	0.027	3	1.020	0.017
	4	3	1.000	0.008	3	0.989	0.005	3	0.991	0.003	3	0.994	0.011
	8	3	1.000	0.003	3	1.000	0.017	3	1.000	0.010	3	0.994	0.013
	12	3	1.000	0.012	3	0.984	0.009	3	0.986	0.011	3	0.988	0.014
	16	3	1.000	0.009	3	1.003	0.004	3	1.006	0.018	3	1.013	0.023
	20	3	1.000	0.105	3	1.069	0.057	3	1.108	0.016	1	1.088	-
	24	3	1.000	0.024	3	1.040	0.027	3	1.019	0.030	3	1.047	0.049
	48	3	1.000	0.142	1	1.124	-	3	0.973	0.040	3	1.362	0.031
	72	3	1.000	0.150	3	0.853	0.176	3	0.872	0.093	3	1.017	0.044
62	1	3	0.968	0.017	3	0.966	0.014	3	0.962	0.009	3	0.972	0.004
	2	3	1.037	0.008	3	1.013	0.020	3	1.020	0.015	3	1.034	0.014
	4	3	1.001	0.003	3	0.982	0.007	3	0.964	0.007	3	0.982	0.021
	8	3	0.977	0.002	3	0.984	0.006	3	0.995	0.016	3	0.953	0.071
	12	3	0.850[a]	0.216	3	0.975	0.011	3	0.973	0.014	3	0.977	0.017
	16	3	1.008	0.009	3	1.011	0.004	3	0.984	0.037	3	1.000	0.022
	20	3	1.051	0.039	3	1.086	0.051	3	0.936	0.188	3	1.100	0.013
	24	3	0.928	0.013	3	0.990	0.019	3	1.042	0.033	3	1.108[b]	0.028
	48	3	1.280[a]	0.051	3	1.301	0.068	1	1.384	-	2	1.342	0.015
	72	3	1.229[a]	0.009	3	1.261	0.022	3	1.281	0.027	3	1.274	0.027

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-9. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	91.6	2.1	3	92.1	2.1	3	91.5	1.9	3	90.7	1.7
	1	3	94.4	0.9	3	94.5	0.8	3	94.2	0.9	3	94.2	0.9
	2	3	92.2	0.5	3	91.2	1.2	3	92.7	0.7	3	90.7	2.4
	4	3	87.3	2.7	3	88.8	1.1	3	87.2	0.5	3	89.3	3.9
	8	3	91.7	0.8	3	87.7	1.8	3	90.6	3.0	3	91.9	0.2
	12	3	93.8	0.4	3	94.0	0.8	3	93.4	0.4	3	91.9	0.6
	16	3	85.3	3.4	3	82.0	3.0	3	81.0	4.4	3	84.0	0.6
	20	3	76.2	4.2	3	73.9	3.0	3	72.0	3.5	3	77.2	1.9
	24	3	83.5	2.7	3	82.1	3.3	3	82.8	3.5	3	77.9	4.6
	48	3	85.7	1.8	3	84.7	1.3	3	86.7	3.7	3	85.7	2.8
101	1	3	93.1	1.1	3	93.3	0.6	3	94.2	0.4	3	94.1	0.6
	2	3	94.1	0.4	3	95.0	0.9	3	95.8	1.9	3	94.1	1.3
	4	3	89.0	2.0	3	89.0	1.9	3	89.3	4.3	3	89.5	1.5
	8	3	95.5	0.7	2	95.3	0.2	3	95.0	0.4	3	94.9	1.0
	12	3	90.3	6.0	3	92.7	0.3	3	81.2	21.7	3	86.5	12.9
	16	3	89.3	2.1	3	88.4	1.6	3	87.7	0.5	3	88.0	0.8
	20	3	87.8	0.9	3	88.8	0.9	3	88.5	1.0	3	87.5	1.5
	24	3	35.3	5.5	3	42.9	4.8	3	47.3	8.6	3	49.6	9.7
	48	3	77.3	6.0	3	87.6	0.9	3	86.0	1.4	3	88.8	1.8

TABLE L-10. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.022	3	1.006	0.023	3	0.999	0.021	3	0.990	0.019
	1	3	1.000	0.010	3	1.001	0.009	3	0.998	0.009	3	0.997	0.009
	2	3	1.000	0.005	3	0.989	0.013	3	1.006	0.008	3	0.983	0.026
	4	3	1.000	0.031	3	1.017	0.012	3	0.999	0.006	3	1.023	0.045
	8	3	1.000	0.009	3	0.955	0.020	3	0.987	0.032	3	1.002	0.002
	12	3	1.000	0.005	3	1.002	0.008	3	0.996	0.004	3	0.980	0.007
	16	3	1.000	0.040	3	0.961	0.036	3	0.949	0.051	3	0.985	0.007
	20	3	1.000	0.055	3	0.970	0.039	3	0.944	0.046	3	1.013	0.025
	24	3	1.000	0.032	3	0.984	0.039	3	0.992	0.042	3	0.933	0.055
	48	3	1.000	0.021	3	0.988	0.015	3	1.012	0.043	3	1.001	0.033
101	1	3	0.986	0.012	3	0.989	0.006	3	0.998	0.004	3	0.997	0.007
	2	3	1.021	0.004	3	1.031	0.009	3	1.038	0.021	3	1.020	0.014
	4	3	1.019	0.023	3	1.020	0.022	3	1.023	0.050	3	1.025	0.017
	8	3	1.041	0.008	2	1.038	0.002	3	1.036	0.005	3	1.034	0.011
	12	3	0.962	0.063	3	0.988	0.003	3	0.865	0.232	3	0.922	0.138
	16	3	1.047	0.025	3	1.036	0.019	3	1.028	0.005	3	1.032	0.009
	20	3	1.152[a]	0.012	3	1.165	0.012	3	1.162	0.013	3	1.148	0.020
	24	3	0.423[a]	0.066	3	0.514	0.057	3	0.566	0.104	3	0.594	0.116
	48	3	0.902[a]	0.070	3	1.023[b]	0.011	3	1.003[b]	0.016	3	1.037[b]	0.021

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-11. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	94.5	2.3	-	-	-	3	95.1	0.7	-	-	-
	1	3	90.6	2.5	3	91.5	2.0	3	90.4	2.1	3	90.7	1.0
	2	3	85.6	2.3	3	83.4	3.6	3	86.4	4.5	3	83.9	2.7
	4	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	8	3	84.2	2.1	3	81.0	0.4	3	83.2	0.5	3	85.5	1.9
	12	3	91.4	0.7	3	89.9	0.4	3	90.7	2.3	3	91.3	1.9
	16	3	94.2	0.6	3	93.4	1.3	3	91.7	0.6	3	93.2	0.5
	20	3	92.3	1.3	3	91.9	0.1	2	90.3	0.4	3	93.4	1.7
	24	3	88.9	0.9	3	88.2	1.2	3	86.4	0.4	3	80.6	1.8
	72	3	71.4	10.8	3	69.6	3.1	3	60.7	6.6	3	81.1	9.5
101	0	-	-	-	3	94.9	0.5	-	-	-	3	93.5	2.0
	1	3	90.3	1.7	3	89.3	4.6	3	90.1	2.3	3	93.2	0.4
	2	3	91.7	0.9	3	92.6	1.2	3	93.1	1.4	3	92.9	1.4
	4	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	8	3	90.3	2.5	3	92.0	1.1	3	92.9	1.4	3	94.3	0.9
	12	3	91.3	1.2	3	91.2	0.7	3	90.9	0.7	3	91.1	0.7
	16	3	89.5	0.9	3	89.8	0.9	3	89.9	0.6	3	92.9	0.5
	20	3	87.6	2.0	3	88.1	2.2	3	88.1	2.2	3	90.2	1.6
	24	3	60.6	8.6	3	23.5	13.2	3	57.5	24.0	3	60.1	13.3
	72	3	38.7	8.8	3	36.8	12.1	3	40.2	13.8	3	53.2	6.3

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

TABLE L-12. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 101 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.025	-	-	-	3	1.006	0.008	-	-	-
	1	3	1.000	0.028	3	1.010	0.022	3	0.998	0.023	3	1.002	0.012
	2	3	1.000	0.027	3	0.974	0.042	3	1.009	0.053	3	0.980	0.032
	4	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	8	3	1.000	0.025	3	0.962	0.005	3	0.988	0.006	3	1.015	0.023
	12	3	1.000	0.007	3	0.983	0.004	3	0.992	0.025	3	0.999	0.021
	16	3	1.000	0.007	3	0.992	0.013	3	0.973	0.006	3	0.989	0.006
	20	3	1.000	0.014	3	0.997	0.002	2	0.979	0.004	3	1.012	0.018
	24	3	1.000	0.010	3	0.993	0.013	3	0.972	0.005	3	0.906	0.020
	72	3	1.000	0.152	3	0.975	0.044	3	0.850	0.093	3	1.135	0.133
101	0	-	-	-	3	1.004	0.005	-	-	-	3	0.990	0.022
	1	3	0.997	0.019	3	0.986	0.050	3	0.995	0.025	3	1.029	0.005
	2	3	1.070	0.011	3	1.082	0.014	3	1.087	0.017	3	1.085	0.017
	4	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	8	3	1.072	0.030	3	1.092	0.013	3	1.104[b]	0.017	3	1.120[b]	0.010
	12	3	0.999	0.013	3	0.998	0.007	3	0.994	0.007	3	0.996	0.008
	16	3	0.950	0.010	3	0.953	0.010	3	0.954	0.006	3	0.986[b]	0.005
	20	3	0.949	0.021	3	0.955	0.024	3	0.955	0.023	3	0.978	0.017
	24	3	0.682[a]	0.097	3	0.264	0.149	3	0.647	0.270	3	0.676	0.149
	72	3	0.542[a]	0.123	3	0.515	0.170	3	0.563	0.193	3	0.745	0.089

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

[a] Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.

[b] Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-13. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	90.8	5.3	3	81.6	5.3	-	-	-	-	-	-
	1	3	89.6	0.7	3	89.6	1.7	3	86.3	1.4	3	86.5	2.6
	2	3	89.3	0.7	3	87.4	3.2	3	87.8	1.4	3	87.0	1.5
	4	3	89.5	1.5	2	91.2	2.2	3	90.1	0.8	3	90.0	1.0
	8	3	82.0	2.8	3	76.9	3.3	3	78.9	3.2	3	79.3	1.4
	12	3	79.0	9.5	3	83.7	3.9	3	86.3	1.6	3	83.8	2.1
	16	3	78.5	9.3	3	81.5	1.3	3	86.7	4.9	3	80.2	6.9
	20	3	88.8	2.3	3	87.4	2.7	3	90.9	1.8	3	91.4	1.8
	24	2	88.2	0.7	3	90.1	1.4	3	89.9	2.3	3	90.9	1.7
	48	3	85.9	3.3	3	83.2	2.7	3	87.7	3.1	3	86.1	5.9
	72	3	82.4	4.7	3	85.7	3.8	3	86.7	2.4	3	87.7	2.7
171	0	3	74.9	2.0	3	83.7	7.1	-	-	-	-	-	-
	1	3	85.0	1.9	3	84.6	1.1	3	84.5	1.0	3	86.0	2.1
	2	3	86.9	2.8	3	87.8	0.9	3	87.4	1.1	3	85.9	0.7
	4	3	86.0	2.7	3	83.7	4.1	3	82.1	5.2	3	82.0	0.9
	8	3	84.4	3.3	3	86.8	0.8	3	84.1	3.6	3	83.0	3.9
	12	3	88.8	4.2	3	87.7	4.6	3	89.8	0.8	3	89.1	1.8
	16	3	80.1	2.6	3	81.7	2.6	3	84.3	2.0	3	81.5	3.4
	20	3	76.4	4.0	3	85.1	7.8	3	86.0	1.9	3	88.6	2.1
	24	3	70.0	1.3	3	73.2	0.8	3	75.6	2.5	3	76.0	2.1
	48	3	36.2	3.0	3	41.3	9.1	3	43.2	3.4	3	47.1	6.8
	72	3	34.7	5.0	3	35.1	3.5	3	37.3	3.2	3	41.4	2.1

TABLE L-14. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE SINGLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.058	3	0.898	0.058	-	-	-	-	-	-
	1	3	1.000	0.008	3	1.000	0.019	3	0.963	0.016	3	0.964	0.029
	2	3	1.000	0.008	3	0.979	0.036	3	0.983	0.016	3	0.974	0.017
	4	3	1.000	0.016	2	1.019	0.024	3	1.006	0.009	3	1.005	0.011
	8	3	1.000	0.034	3	0.937	0.041	3	0.961	0.039	3	0.966	0.017
	12	3	1.000	0.121	3	1.059	0.050	3	1.093	0.021	3	1.061	0.027
	16	3	1.000	0.119	3	1.038	0.017	3	1.105	0.063	3	1.022	0.087
	20	3	1.000	0.026	3	0.985	0.031	3	1.024	0.020	3	1.029	0.020
	24	2	1.000	0.008	3	1.021	0.015	3	1.018	0.026	3	1.030	0.019
	48	3	1.000	0.039	3	0.968	0.031	3	1.020	0.036	3	1.002	0.068
	72	3	1.000	0.057	3	1.040	0.047	3	1.052	0.029	3	1.064	0.033
171	0	3	0.825	0.022	3	0.922	0.078	-	-	-	-	-	-
	1	3	0.949	0.021	3	0.944	0.013	3	0.943	0.012	3	0.959	0.023
	2	3	0.973	0.032	3	0.983	0.011	3	0.979	0.012	3	0.962	0.008
	4	3	0.961	0.031	3	0.935	0.045	3	0.917	0.058	3	0.916	0.010
	8	3	1.029	0.041	3	1.058	0.009	3	1.025	0.043	3	1.011	0.047
	12	3	1.125[a]	0.053	3	1.111	0.058	3	1.137	0.010	3	1.128	0.023
	16	3	1.021	0.033	3	1.042	0.033	3	1.075[b]	0.025	3	1.039	0.044
	20	3	0.861[a]	0.045	3	0.958[b]	0.088	3	0.969[b]	0.021	3	0.998[b]	0.023
	24	3	0.794[a]	0.015	3	0.830	0.009	3	0.857	0.028	3	0.861	0.024
	48	3	0.421[a]	0.035	3	0.481	0.106	3	0.503	0.039	3	0.548	0.079
	72	3	0.421[a]	0.060	3	0.426	0.043	3	0.452	0.038	3	0.502	0.026

[a]Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.
[b]Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

TABLE L-15. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Percent of PI Negative Cells) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	92.1	3.5	3	91.9	2.7	-	-	-	-	-	-
	12	3	95.8	0.5	3	95.0	1.0	3	95.2	1.0	3	94.0	1.2
	16	3	91.4	3.2	3	92.8	2.4	3	91.3	4.2	3	87.8	3.7
	20	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	24	3	91.2	0.2	3	92.8	1.4	3	87.7	8.5	3	93.3	3.6
	48	3	94.0	3.1	3	91.5	1.1	3	92.5	1.6	3	94.8	0.6
	72	3	78.8	5.2	3	78.0	4.4	3	80.6	3.1	3	92.2	4.4
171	12	3	96.4	1.1	3	95.7	0.7	3	95.5	2.2	3	96.8	0.4
	16	3	91.0	2.9	3	92.6	0.5	3	92.5	1.1	3	93.5	1.0
	20	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	24	3	63.5	5.6	3	71.8	6.6	3	73.1	5.5	3	76.8	5.9
	48	3	61.0	8.7	3	58.4	13.9	3	86.4	16.2	3	71.4	4.9
	72	3	30.3	2.0	3	28.1	1.5	3	34.6	2.3	3	37.5	7.8

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

TABLE L-16. DESCRIPTIVE STATISTICS FOR CELL VIABILITY (Fraction of Vehicle Control) WITH NIACIN (NI) ADDITION USING THE MULTIPLE ADDITION MODE AND 171 μM HD

HD Conc. (μM)	Time (hrs)	NI = 0 (mM)			NI = 0.1 (mM)			NI = 1 (mM)			NI = 10 (mM)		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
0	0	3	1.000	0.038	3	0.998	0.029	-	-	-	-	-	-
	12	3	1.000	0.005	3	0.992	0.010	3	0.994	0.011	3	0.981	0.013
	16	3	1.000	0.035	3	1.016	0.026	3	0.999	0.045	3	0.961	0.041
	20	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	24	3	1.000	0.003	3	1.018	0.016	3	0.962	0.093	3	1.023	0.039
	48	3	1.000	0.033	3	0.972	0.012	3	0.984	0.017	3	1.008	0.007
	72	3	1.000	0.066	3	0.991	0.055	3	1.024	0.039	3	1.170	0.055
171	12	3	1.007	0.011	3	0.999	0.007	3	0.997	0.022	3	1.011	0.004
	16	3	0.996	0.032	3	1.014	0.005	3	1.012	0.012	3	1.023	0.011
	20	0 *	-	-	0 *	-	-	0 *	-	-	0 *	-	-
	24	3	0.696[a]	0.061	3	0.787[b]	0.072	3	0.801[b]	0.061	3	0.842[b]	0.065
	48	3	0.648[a]	0.093	3	0.621	0.148	3	0.919[b]	0.173	3	0.759	0.052
	72	3	0.385[a]	0.025	3	0.356	0.019	3	0.440	0.029	3	0.476[b]	0.099

*Sample mean excluded from descriptive statistics due to corresponding vehicle control mean being outside of control limits.

[a] Differs ($p \leq 0.05$) from vehicle-control (HD = 0, NI = 0) value.

[b] Differs ($p \leq 0.05$) from HD-control (HD-exposed, NI = 0) value.

APPENDIX M

SUMMARY STATISTICS OF CELL NUMBER, PROTEIN CONTENT, AND CYTOTOXICITY PARAMETERS

TABLE M-1. SUMMARY FOR HD-INDUCED ALTERATION OF PARAMETER RESPONSES NORMALIZED BY THE TIME ZERO PARAMETER VALUE*

Parameter	Time (hrs)	Run	HD Concentration Control	13 μM	62 μM	101 μM	171 μM
Total Protein (μg)	2	1	0.72	0.60	0.39	0.86	0.56
		2	0.73	0.51	0.26	0.40	0.57
		3	1.03	0.71	1.04	1.05	1.12
	24	1	0.91	1.10	0.88	0.60	0.35
		2	1.66	1.45	0.74	0.67	0.38
		3	1.25	1.47	1.08	0.96	0.55
	48	1	1.07	1.02	0.90	0.55	0.27
		2	2.17	1.69	1.65	1.56	0.53
		3	1.02	1.24	0.94	0.82	0.48
	72	1	1.64	1.30	1.56	1.18	0.35
		2	3.12	2.16	1.54	1.17	0.39
		3	1.32	1.36	1.14	0.96	0.43
Total Cell Count ($x10^5$)	2	1	1.59	1.47	0.92	1.15	0.91
		2	0.80	0.45	0.74	0.49	0.49
		3	1.16	0.76	0.95	0.99	0.78
	24	1	0.74	1.10	0.91	0.64	0.77
		2	1.47	0.92	0.52	0.38	0.49
		3	1.60	0.86	0.92	0.85	0.51
	48	1	1.16	0.94	0.76	0.71	0.72
		2	2.45	0.45	0.61	0.49	0.63
		3	1.14	0.79	0.47	0.32	0.92
Total Cell Count ($x10^5$)	72	1	1.44	0.89	0.84	0.56	0.83
		2	3.00	0.47	0.56	0.38	0.40
		3	1.58	0.62	0.83	0.57	0.50

TABLE M-1.
(Continued)

Parameter	Time (hrs)	Run	HD Concentration Control	13 μM	62 μM	101 μM	171 μM
Viable Cell Count ($x10^5$)	2	1	1.52	1.34	0.89	1.09	0.83
		2	0.74	0.39	0.51	0.43	0.45
		3	1.14	0.68	0.90	0.94	0.67
	24	1	0.68	1.08	0.90	0.57	0.54
		2	1.50	0.79	0.42	0.32	0.06
		3	1.37	0.72	0.78	0.77	0.35
	48	1	0.78	0.86	0.70	0.62	0.21
		2	2.27	0.42	0.50	0.42	0.05
		3	0.84	0.67	0.42	0.29	0.44
	72	1	1.00	0.77	0.83	0.44	0.02
		2	2.90	0.38	0.49	0.30	0.02
		3	1.25	0.47	0.70	0.54	0.26
PI Negative	2	1	0.96	0.91	0.97	0.95	0.93
		2	0.92	0.86	0.73	0.86	0.91
		3	0.98	0.89	0.94	0.94	0.85
	24	1	0.93	0.98	0.99	0.90	0.69
		2	1.02	0.86	0.83	0.84	0.13
		3	0.86	0.83	0.85	0.90	0.67
	48	1	0.66	0.90	0.91	0.87	0.28
		2	0.93	0.93	0.82	0.86	0.07
		3	0.67	0.85	0.91	0.90	0.43
	72	1	0.71	0.87	0.98	0.80	0.02
		2	0.97	0.79	0.87	0.78	0.04
		3	0.80	0.75	0.79	0.94	0.50

TABLE M-1.
(Continued)

Parameter	Time (hrs)	Run	HD Concentration Control	13 μM	62 μM	101 μM	171 μM
ng Protein/Cell	2	1	0.45	0.40	0.47	0.78	0.79
		2	0.94	1.20	0.44	0.82	1.23
		3	0.91	0.94	1.06	1.03	1.40
	24	1	1.22	0.99	0.96	0.93	0.46
		2	1.15	1.56	1.54	1.75	0.82
		3	0.77	1.66	1.19	1.11	1.05
	48	1	0.99	1.08	1.16	0.76	0.38
		2	0.91	4.24	2.84	3.28	0.87
		3	1.00	1.53	1.93	2.51	0.56
	72	1	1.13	1.51	1.84	2.13	0.42
		2	1.03	4.67	2.76	3.27	0.97
		3	0.83	2.20	1.33	1.63	0.89

* Values are parameter responses normalized to the time-zero response value. Numbers are averages of at least duplicate observations.

APPENDIX N

POLY(ADP-RIBOSE) POLYMERASE DATA

TABLE N-1. PADPRP ASSAY WITH HUMAN KERATINOCYTES (STRAIN #732)

PADPRP Determination: April 5, 1994

Sample No.	Inc. (min.)	DNase	CPM	CPM-BG	DPM	pmol NAD Inc.	pmol per mg prot.	Mean	STD
1	5	-	817	498	1364	2.1	11.3	11.1	1.52
2	5	-	733	414	1133	1.7	9.4		
3	5	-	865	546	1495	2.3	12.4		
4	5	+	2241	1922	5266	8.0	43.8	38.2	6.65
5	5	+	2066	1747	4787	7.2	39.8		
6	5	+	1672	1353	3705	5.6	30.8		
7	2	-	413	94	256	0.4	2.1	1.23	1.1
8	2	-	388	69	188	0.3	1.6		
9	2	-	298	0	0	0.0	0.0		
10	2	+	736	417	1142	1.7	9.5	11.3	4.28
11	2	+	1031	712	1951	3.0	16.2		
12	2	+	682	363	995	1.5	8.3		
11	BG	-	357						
12	BG	-	281						

Protein Determination:

BSA STD UG/ML	OD570 Value	-BG
0	0.088	0.000
0	0.088	0.000
10	0.096	0.008
10	0.094	0.006
31.25	0.110	0.022
31.25	0.109	0.021
62.5	0.137	0.049
62.5	0.135	0.047
125	0.191	0.103
125	0.159	0.071
250	0.216	0.128
250	0.245	0.157
500	0.378	0.290
500	0.404	0.316

Regression Output:

Y-Int.		0.004
SE of Y-Int		0.012
R Squared		0.987
No. of Observations		14
df		12
slope (m)	0.000594	
SE of slope	0.000019	

Description	Dil.	OD570 Value	OD570 -BG	[PROT] mg/mL	mg Protein Per PADPRP Assay ------ Value	----- Average
BG	-	0.091				
BG	-	0.087				
Sample	30	0.206	0.117	5.71	0.171	0.182
Sample	30	0.220	0.131	6.42	0.193	

TABLE N-2. PADPRP ASSAY WITH HUMAN KERATINOCYTES (STRAIN #732)

PADPRP Determination: April 6, 1994

Sample No.	Inc. (min.)	DNase	3-AB (1 mM)	CPM	CPM-BG	DPM	pmol NAD Inc.	pmol per mg prot.	Mean	STD
1	5	-	-	325	98	267	0.4	2.7	1.73	1.28
2	5	-	-	307	80	220	0.3	2.2		
3	5	-	-	237	10	28	0.0	0.3		
4	5	+	-	1351	1124	3078	4.7	31.1	34.3	3.26
5	5	+	-	1460	1233	3379	5.1	34.1		
6	5	+	-	1586	1359	3724	5.6	37.6		
7	5	+	+	253	26	71	0.1	0.7	0.35	0.36
8	5	+	+	201	-26	0	0.0	0.0		
9	5	+	+	239	12	33	0.1	0.3		
10	BG	-		276						
11	BG	-		178						

Protein Determination:

BSA STD UG/ML	OD570 Value	OD570 -BG
0	0.076	-0.004
0	0.084	0.004
10	0.097	0.017
10	0.096	0.016
31.25	0.118	0.038
31.25	0.122	0.042
62.5	0.153	0.073
62.5	0.158	0.078
125	0.227	0.147
125	0.205	0.125
250	0.306	0.226
250	0.309	0.229
500	0.491	0.411
500	0.520	0.440

Regression Output:

Y-int. (B)		0.015
Std Err of Y Est		0.013
R Squared		0.992
No. of Observations		14
DF		12
Slope (m)	0.000836	
SE of Slope	0.000021	

Description	Dil.	OD570	OD570 - BG	[PROT] mg/mL	mg Protein Per PADPRP Assay: Value	mg Protein Per PADPRP Assay: Average
BG	-	0.088				
BG	-	0.085				
Sample	20	0.296	0.210	4.66	0.140	0.150
Sample	20	0.324	0.238	5.33	0.160	

TABLE N-3. PADPRP ASSAY WITH HUMAN KERATINOCYTES (STRAIN #732)
EFFECT OF 3-AMINOBEZAMIDE PREINCUBATION

PADPRP Determination: April 13, 1994

Sample No.	Inc. (min)	DNase	3-AB (1 mM)	CPM	CPM-BG	DPM	pmol NAD Inc.	pmol per mg prot.	Mean	STD
1	5	-	-	766	489	1338	2.0	9.4	10.2	1.21
2	5	-	-	880	603	1651	2.5	11.6		
3	5	-	-	777	500	1369	2.1	9.7		
4	5	+	-	3387	3110	8520	12.9	65.6	51.4	13.0
5	5	+	-	2572	2295	6287	9.5	48.4		
6	5	+	-	2180	1903	5213	7.9	40.2		
7	5	+	+	733	456	1248	1.9	9.5	18.1	7.73
8	5	+	+	1251	974	2668	4.0	20.3		
9	5	+	+	1451	1174	3215	4.9	24.5		
10	BG	-		205						
11	BG	-		349						

Protein Determination:

BSA STD UG/ML	OD570 Value	OD570 -BG
0	0.081	-0.002
0	0.085	0.002
10	0.090	0.007
10	0.099	0.016
31.25	0.120	0.037
31.25	0.132	0.049
62.5	0.162	0.079
62.5	0.153	0.070
125	0.215	0.132
125	0.211	0.128
250	0.297	0.214
250	0.304	0.221
500	0.487	0.404
500	0.518	0.435

Regression Output:

y-int (b)	0.013
Std Err of Y Est	0.013
R Squared	0.993
No. of Observations	14
Degrees of Freedom	12
Slope (m)	0.000821
SE of Slope	0.00002

Description	Dil.	OD570 Value	OD570 -BG	[PROT] mg/mL	mg Protein Per PADPRP Assay Value	Average
BG	-	0.093				
BG	-	0.088				
Sample 1 to 3	30	0.293	0.203	6.92	0.207	0.215
Sample 1 to 3	30	0.306	0.216	7.39	0.222	
Sample 4 to 6	30	0.288	0.198	6.73	0.202	0.197
Sample 4 to 6	30	0.278	0.188	6.37	0.191	
Sample 7 to 9	30	0.288	0.198	6.73	0.202	0.199
Sample 7 to 9	30	0.282	0.192	6.51	0.195	

TABLE N-4. PADPRP ASSAY WITH HUMAN KERATINOCYTES (STRAIN #732) COMPARISION OF SONICATION AND SOLUBILIZATION

PADPRP Determination: April 15, 1994

Sample No.	Inc. (min.)	Sonication	Solubilization	CPM	CPM-BG	DPM	pmol NAD Inc.	pmol per mg. prot.	Mean	STD
1	5	-	+	1079	778	2132	3.2	15.8	11.0	4.60
2	5	-	+	628	327	897	1.4	6.7		
3	5	-	+	823	522	1429	2.2	10.6		
4	5	+	-	722	421	1153	1.7	6.6	4.83	2.16
5	5	+	-	455	154	423	0.6	2.4		
6	5	+	-	645	344	942	1.4	5.4		
7	5	+	+	1303	1002	2746	4.2	15.8	6.26	8.40
8	5	+	+	226	0	0	0.0	0.0		
9	5	+	+	490	189	518	0.8	3.0		
10	BG	-	-	312						
11	BG	-	-	290						

Protein Determination:

BSA STD UG/ML	OD570 Value	OD570 -BG
0	0.098	0.004
0	0.090	-0.004
10	0.109	0.015
10	0.114	0.020
31.25	0.139	0.045
31.25	0.133	0.039
62.5	0.170	0.076
62.5	0.170	0.076
125	0.231	0.137
125	0.238	0.144
250	0.352	0.258
250	0.357	0.263
500	0.558	0.464
500	0.572	0.478

Regression Output:

Y-Int (b)	0.013
Std Err of Y Est	0.011
R Squared	0.996
No. of Observations	14
Degrees of Freedom	12
Slope (m)	0.000935
SE of Slope	0.000017

Description	Dil.	OD570	OD570 -BG	[PROT] mg/mL	mg Protein Per PADPRP Assay: Value	Average
BG	-	0.100				
BG	-	0.103				
Solubilized Sample	30	0.319	0.218	6.55	0.197	0.204
Solubilized Sample	30	0.334	0.233	7.04	0.211	
Sonicated Sample	30	0.389	0.288	8.80	0.264	0.263
Sonicated Sample	30	0.387	0.286	8.74	0.262	